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TITLE OF THE INVENTION (500 characters max)

HUMAN MAST CELL-EXPRESSED MEMBRANE PROTEIN

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Respectfully submitted,

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U.S. PROVISIONAL
PATENT APPLICATION
FOR:

HUMAN MAST CELL-EXPRESSED MEMBRANE PROTEIN

BY

KANG LI, SHEN-WU WANG,

GUANGHUI HU AND ZHENGBIN YAO

FIELD OF THE INVENTION

The present invention relates to purinergic receptor-like polypeptides in mast cells.

BACKGROUND OF THE INVENTION

A number of purinergic receptors for extracellular nucleotides have been reported. These P2 purinergic receptors are a class of G protein-coupled receptors activated primarily by ATP, ADP, UTP, and UDP. P2 receptors can be subdivided into two classes: P2X receptors (ion channels) and P2Y receptors (G-protein coupled receptors). Within the P2Y family, five functional human receptors (P2Y1, 2, 4, 6 & 11) have been identified. In addition, a number of uncloned purinergic receptors have been postulated based on specific pharmacology, the most well known of these being the P2T receptor, currently classified within the P2Y family, which is found on human platelets. Purinergic-like orphan receptors (P2Y5, 9 and 10) have also been postulated based on protein homology but to date these remain unresponsive to a wide range of purinergic ligands.

P2Y receptors are 7-membrane-spanning proteins ranging in size from 328 to 379 amino acids and a molecular mass between 41 and 53 kD after glycosylation. The aminoterminal end faces the extracellular environment and the carboxy-terminal is on the cytoplasmic side of the plasma membrane.

Purinergic receptors are known to be involved in neurotransmission, ADP-induced platelet shape change and aggregation, lung mucociliary clearance and relaxation of smooth muscle. Purinergic receptors have also been suggested to have roles in the cardiovascular system, gastro-intestinal (GI) tract, immune system and endocrine system.

Asthma is a disease that is characterized by inflammation, hyperresponsiveness, and remodeling of the airways. The main therapeutic drugs for the treatment of asthma are β2-agonists (which provide symptomatic relief from airway narrowing) and corticosteroids (which aim to reduce the inflammatory process in the lung and to improve or reduce further deterioration of lung function). Despite these and other forms of treatment, asthma morbidity is on the rise as is its worldwide prevalence.

Human mast cells play a central role in the development of asthma, allergic and inflammatory diseases by releasing cytokines, chemokines, proteases and

small molecule mediators (Nechushtan, H. and Razin, E. 1996 Critical Review Oncology/Hematology 23:131-150; Bradding, P. and Halgate, S. 1999 Critical Review Oncology/Hematology 31:119-133; Wedemeyer et al., 2000 Cur. Opinion In Immunol. 12:624-631; Hart, PH, 2000 Immunol. Cell Biol. 79:149-153; Lee, DM et al., 2002 Science 297:1689; Boyce, J. 2003 J. Allergy Clin. Immunol., 111:24-32). For example, in asthmatic patients, the exacerbation of symptoms was accompanied with gene activation, de novo synthesis and release of cytokines and chemokines from the mast cell, which cause exaggerated bronchoconstriction, induce human airway smooth muscle cell proliferation, and recruit and activate inflammatory cells. Moreover, the number of mast cells present on asthmatic airway smooth muscle is increased compared with that on non-asthmatic airway smooth muscle.

The stimuli (such as allergen-induced IgE cross-linking) that caused mast cell activation and asthma are linked to Increase of intracellular calcium concentration and calcium-mediated signaling pathways, which enhance expression and de novo synthesis of cytokines, chemokines and proteases (Nature 1999, 402:B24; Immunity 2002, 16:441). Nuclear factor of activated T-cell (NFAT) and activator protein-1 (AP-1) are the transcription factors that play important role in the signaling cascade of mast cells (J. Biol. Chem. 1995, 270:16333; Annu. Rev. Immunol. 1997, 15:707; PNAS 2003, 100:1169). Therefore, activation of calcium-mediated pathways, transcription factors (NFAT and AP-1) and asthma-related cytokine, chemokine and protease genes may be used as a proxy for identifying therapeutic target in mast cells.

SUMMARY OF THE INVENTION

The present invention relates to a method of screening for a compound that modulates of GPR91 receptor activity comprising (a) preparing a transfected cell comprising a nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO 2; (b) contacting transfected cell(s) with at least one compound whose ability to modulate the GPR91 receptor activity is sought to be determined; and (c) monitoring said cell for a compound that modulates the receptor's activity.

The cells may be stably or transiently transfected. The cells may be Mast cells, knockout cells isolated from GPR91-knockout mice (GPR91$^-$/GPR91$^-$), CHO,

COS-7, 293 cells, HELA, and cell lines established from mast cells or leukemic mast cells.

The modulation of GPR91 activity may be monitored by measuring intracellular cacium flux, measuring the level of expression of a reporter gene, such as an NFAT-Luc reporter plasmid, measuring cytokine expression levels, and other common methods of measuring activation or inhibition of a receptor. The reporter gene may be operatively linked to a GPR91 responsive transcription element, such as a NFAT (Nuclear Factor of Activating T-cell) binding motif element.

The present invention also includes a method of screening for agonists or antagonists of GPR91 activity comprising: (a) contacting cells which express a GPR91 receptor with a candidate compound, (b) assaying a cellular response, and (c) comparing the cellular response to a standard cellular response made in absence of the candidate compound; whereby, an increased cellular response over the standard indicates that the compound is an agonist and a decreased cellular response over the standard indicates that the compound is an antagonist.

The present invention relates to agonists and antagonists, including those that provide therapeutic benefit in treating immune diseases, including allergic diseases, such as asthma, as well as inflammatory diseases. In one embodiment, antagonists are a soluble form of GPR91 receptors and soluble polypeptides derived from the extracellular domains of GPR91 receptors that are capable of binding the GPR91 receptor. The antagonists may be peptides selected from the extracellular domain of SEQ ID NO:2 or antagonist fragments thereof. These peptides block the binding of the natural ligand for GPR91 by binding to the ligand and preventing the ligand from binding to the native receptor.

The present invention includes antibodies that specifically bind GPR91. These antibodies may be neutralizing, agonists or antagonists. Included are antibodies that arepolyclonal or monoclonal, which may be humanized, human, chimeric, bispecific, or conjugated.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

The term "purified polypeptide" means a polypeptide identified and separated from at least one contaminant polypeptide ordinarily associated with the

purified polypeptide in its native environment, particularly a polypeptide separated from its cellular environment.

The term "isolated polynucleotide" means a polynucleotide identified and separated from at least one contaminant polynucleotide ordinarily associated with the isolated polynucleotide in its native environment, particularly a polynucleotide separated from its cellular environment.

The term "native" when used to describe a polynucleotide, polypeptide sequence, or other molecule means a polypeptide, polynucleotide, or other molecule as found in nature, e.g., a polypeptide or polynucleotide sequence that is present in an organism such as a virus, or prokaryotic or eukaryotic cell that can be isolated from a source in nature and that has not been intentionally modified to change is structure, properties, or function. An unisolated cellular polynucleotide having the nucleotide sequence shown in SEQ ID NO:1 is a native polynucleotide and an unpurified cellular polypeptide having the amino acid sequence shown in SEQ ID NO:2 is a native polypeptide.

The term "percent sequence identity" means the percentage of sequence similarity found in a comparison of two or more nucleotide or amino acid sequences. Percent identity can be determined electronically, e.g., by using the MEGALIGN program (DNASTAR, Inc., Madison Wisconsin.). The MEGALIGN program creates alignments between two or more sequences according to different methods, e.g., the clustal method. (See, e.g., Higgins, D. G. and P. M. Sharp (1988) Gene 73:237-244.) The clustal algorithm groups sequences into clusters by examining the distances between all pairs. The clusters are aligned pairwise and then in groups. The percentage similarity between two amino acid sequences, e.g., sequence A and sequence B, is calculated by dividing the length of sequence A, minus the number of gap residues in sequence A, minus the number of gap residues in sequence B, into the sum of the residue matches between sequence A and sequence B, times one hundred. Gaps of low or of no similarity between the two amino acid sequences are not included in determining percentage similarity. Percent identity between nucleotide sequences is counted or calculated by methods known in the art, e.g., the Jotun Hein method given in Hein, J. (1990) Methods Enzymol. 183:626-645. Identity between sequences can

also be determined by other methods known in the art, e.g., by varying hybridization conditions.

The term "variant" when used to describe a polynucleotide sequence means a nucleotide sequence that differs from its native counterpart by one or more nucleotides and either has the same or similar biological function as its native counterpart or does not have the same or similar biological function as its native counterpart but is useful as a probe to identify or isolate its native counterpart. Variants include nucleotide sequences having at least 85 percent sequence identity when compared to its native counterpart, or at least 90 to 95 percent sequence identity, or at least 99 percent sequence identity, and nucleotide sequences that bind to native sequences or their complement under stringent conditions. Variants may include nucleotide sequences that code for the same amino acid sequence as its native counterpart but differ from the native nucleotide sequence based only on the degeneracy of the genetic code.

The term "variant" when used to describe a polypeptide sequence means an amino acid sequence that differs from its native counterpart by one or more amino acids, including modifications, substitutions, insertions, and deletions, and either has the same or similar biological function as its native counterpart or does not have the same or similar biological function as its native counterpart but is useful as an immunogen to produce antibodies that bind to its native counterpart or as an agonist or antagonist for its native counterpart. Variants include polypeptides having at least 70 percent sequence identity when compared to its native counterpart, at least 85 percent sequence identity, and or at least 95 percent sequence identity. Variants include polypeptides with conservative amino acid substitutions.

The term "fragment" when used to describe a polynucleotide means a nucleotide sequence subset of its native counterpart that binds to its native counterpart or its complement under stringent conditions. Preferred fragments have a nucleotide sequence of at least 25 to 50 consecutive nucleotides of the native sequence. Most preferred fragments have an amino acid sequence of at least 50 to 100 consecutive nucleotides of the native sequence.

The term "fragment" when used to describe a polypeptide means an amino acid sequence subset of its native counterpart that either retains any biological

activity of its native counterpart or acts as an immunogen capable of producing an antibody that binds to its native counterpart. Fragments include amino acid sequences of at least 10 to 20 consecutive amino acids of the native sequence or of at least 20 to 30 consecutive amino acids of the native sequence.

The term "agonist" means any molecule that directly or indirectly promotes, enhances, or stimulates the normal function of the GPR91 receptor. One type of agonist is a molecule that interacts with the GPR91 receptor in a way that mimics its ligand, including, but not limited to, an antibody or antibody fragment.

The term "antagonist" means any molecule that blocks, prevents, inhibits, or neutralizes the normal function of the GPR91 receptor. One type of antagonist is a molecule that interferes with the interaction between GPR91 receptor and its ligand, including, but not limited to, an antibody or antibody fragment. Another type of antagonist is an antisense nucleotide that inhibits proper transcription of native GPR91 receptor or siRNA that binds to the native transcript.

The term "conservative amino acid substitution" means that an amino acid in a polypeptide has been substituted for with an amino acid having a similar side chain. For example, glycine, alanine, valine, leucine, and isoleucine have aliphatic side chains; serine and threonine have aliphatic-hydroxyl side chains; asparagine and glutamine have amide-containing side chains; phenylalanine, tyrosine, and tryptophan have aromatic side chains; lysine, arginine, and histidine have basic side chains; and cysteine and methionine have sulfur-containing side chains. Preferred conservative amino acids substitutions are valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "antisense" as used herein, refers to any composition containing nucleotide sequences which are complementary to a specific DNA or RNA sequence. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. Antisense molecules include peptide nucleic acids and may be produced by any method including synthesis or transcription. Once introduced into a cell, the complementary nucleotides combine with natural sequences produced by the cell to form duplexes and block either transcription or translation. The designation "negative" is sometimes used in reference to the antisense strand, and "positive" is sometimes used in reference to the sense strand.

The term "knockout" refers to partial or complete reduction of the expression of at least a portion of a polypeptide encoded by an endogenous gene (such as the GPR91 receptor) of a single cell, selected cells, or all of the cells of a mammal. The mammal may be a "heterozygous knockout" having one allele of the endogenous gene disrupted or "homozygous knockout" having both alleles of the endogenous gene disrupted.

This invention is not limited to the particular methodology, protocols, cell lines, vectors, and reagents described herein because they may vary. Further, the terminology used herein is for the purpose of describing particular embodiments only and is not intended to limit the scope of the present invention. As used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise, e.g., reference to "a host cell" includes a plurality of such host cells.

Because of the degeneracy of the genetic code, a multitude of nucleotide sequences encoding the GPR91 polypeptides of the present invention may be produced. Some of these sequences will be highly homologous and some will be minimally homologous to the nucleotide sequences of any known and naturally occurring nucleotide sequence. The present invention contemplates varying the nucleotide sequence made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the nucleotide sequence that codes for naturally occurring GPR91 receptor and all such variations are to be considered as being specifically disclosed.

Unless defined otherwise, all technical and scientific terms and any acronyms used herein have the same meanings as commonly understood by one of ordinary skill in the art in the field of the invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice of the present invention, the methods, devices, and materials are described herein.

All patents and publications mentioned herein are incorporated herein by reference to the extent allowed by law for the purpose of describing and disclosing the proteins, enzymes, vectors, host cells, and methodologies reported therein that might be used with the present invention. However, nothing herein is to be

construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of the invention and the Examples included herein. The G-protein receptor 91(GPR91) shares significant sequence homology with purinergic receptors, the P2Y family, and with the cysteinyl leukotriene receptors. GPR91 was found to be differentially expressed in human primary mast cells as compared with other cell types, GPR91 was also expressed in human monocytes, and GPR91 was found to activate intracellular calcium flux and calcineurin-mediated signaling pathway when over-expressed in a human cell line derived from mastoma tissue - Human Mast Cell-1 (HMC-1) cells. GPR91 may, therefore, play an important role in stimulating mast cell activities in airway, and/or peripheral or connective tissues for inflammatory and allergic responses. Thus, GPR91 may be used as a therapeutic target for treating mast cell mediated diseases such as allergic and nonallergic astham, chronic obstructive pulmonary disease (COPD), allergic rhinitis, anaphylaxis, allergic gastrointestinal dis disease, atopic dermatitis, rheumatoid arthritis, system sclerosis, and other allergic, autoimmune and inflammatory diseases. Activators/inhibitors of GPR91 for this treatment can be antibodies, peptide mimetics for the ligand, small molecules, antisense, or RNAi.

IDENTIFICATION OF GPR91

The genes differentially expressed in human mast cells were identified initially by comparing the mRNA expression levels among mast cells (cultured from umbilical cord blood CD34+ cells), PBMC (peripheral blood mononuclear cells), and THP-1 (acute monocytic leukemia; lymphocytes) using the gene microarray technology. (See examples) The differential expression of GPR91 in mast cells was further confirmed by quantitative RT-PCR using a number of different cell types and humans tissues.

The BLAST sequence similarity search against the public GenBank database revealed that CAGM2/GPR91 shares significant homology to P2Y purinergic receptors and cysteinyl leukotriene receptors. For examples, the pair-wised comparison using the computer algorism, DNAStar, showed that GPR91 shares 29.9% to 35.7% of amino acid sequence identity to the six known

members of P2Y receptor family, and that it also shares 29.3% to 31.6% of the sequence identity to the cysteinyl leukotriene receptors, CysLT1 and CysLT2. Prediction of the coupling specificity of CAGM2/GPR91 to its G protein indicated that it is most likely involved in interacting with Gαq/11 subtype. This prediction was based on the fact that the specificity of the receptor-G protein interaction is governed by the intracellular domains of the receptor. A data-mining approach combining pattern discovery with membrane topology prediction was used to find patterns of amino acid residues in the intracellular domains of GPCR sequences that are specific for coupling to a particular functional class of G proteins.

To identify the function of GPR91, we performed transient transfection and luciferase reporter assays using the plasmid constructs that express the wild type (wt) of GPR91 (pGPR91), or tagged GPR91 (pGPR91-V5 and pGPR91-20Flag). In HMC-1 cells, the wt GPR91 activated the NFAT (nuclear factor of activating T-cell) promoter-linked luciferase reporter by about 19.8 fold, and it also activated the AP1-promoter-linked luciferase reporter by about 5.3 fold. The activation of NFAT promoter by GPR91 was further verified by applying the specific inhibitor (cyclosporin A) to calcineurin which mediates intracellular signal transduction from calcium influx to NFAT activation. The stimulatory effect to NFAT activation by GPR91 was almost completely blocked by cyclosporin A. These results indicate that GPR91 activates the transcription factor NFAT through a well-known intracellular signaling pathway, i.e., GPR91 can recruit the Gαq/11 (or q/11-like) subunit, activating the G protein, which in turn activates phospholipase C β and results in intracellular calcium flux and NFAT and AP1 activation.

We further identified a group of genes whose promoters were activated by the constitutively active insertion mutant, GPR91-20Flag. The luciferase reporter assay showed that GPR91-20Flag enhanced promoter activaties of IL8, IL13, TNFα, tryptase β1 and tryptase β2 (EXAMPLE 5), which are examples of cytokines, proteases and chemokines that are released from mast cells and involved exacerbation of asthmatic symptoms, allergic and inflammatory diseases (Annu. Rev. Immunol. 1999, 17:255; Am. J. Rspir. Cell Mol. Biol. 1996, 15: 473; J. Immunol. 2002, 168:2603; J. Immunol. 2002, 169:2662; J. Immunol. 2000, 165:7215; Proc. Natl. Acad. Sci. USA 1992, 89:8542; Eur. Respir. J. 2001, 34:50s)

Since it is well documented in the published literature that the intracellular calcium flux via the calcium-mediated signaling pathway is one of the pivotal conditions to activate mast degranulation and cytokine secretion, our findings suggest that GPR91 may play a critical role in the development of asthma, allergy, and other inflammatory diseases. Therefore, GPR91 can be used as a therapeutic target for generating antibodies, screening for small molecule and/or RNAi for the treatment of asthma, allergy, autoimmune diseases, and other inflammatory diseases.

AGONISTS AND ANTAGONISTS

The present invention provides agonists and antagonists that directly or indirectly activate or inhibit the expression or action GPR91. Types of agonist and antagonists include, but are not limited to, polypeptides, proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleotides, organic molecules, bioorganic molecules, peptidomimetics, pharmacological agents and their metabolites, and transcriptional and translation control sequences.

In one embodiment, antagonists are a soluble form of GPR91 receptors and soluble polypeptides derived from the extracellular domains of GPR91 receptors that are capable of binding the GPR91 receptor. The antagonists may be peptides selected from the extracellular domain of SEQ ID NO:2 or antagonist fragments thereof. These peptides block the binding of the natural ligand for GPR91 by binding to the ligand and preventing the ligand from binding to the native receptor.

The agonists and antagonists include antibodies that bind specifically to GPR91 and influence biological actions and functions, e.g., to activate or inhibit the production of cytokines. The antibodies can be polyclonal or monoclonal, and may be chimeric, human, humanized, or deimmunized.

Agonist antibodies may be used to prevent or treat diseases characterized by relatively low cytokine or receptor expression compared to non-disease states. Antagonist antibodies are used to prevent or treat diseases characterized by relatively high cytokine or receptor expression compared to non-disease states.

The agonists and antagonists are used for the treatment of various immune diseases, including, but not limited to allergic diseases such as asthma, allergic

rhinitis, atopic dermatitis, food hypersensitivity and urticaria; transplantation associated diseases including graft rejection and graft-versus-host-disease; autoimmune or immune-mediated skin diseases including bullous skin diseases, erythema multiform and contact dermatitis, psoriasis; rheumatoid arthritis, juvenile chronic arthritis; inflammatory bowel disease (i.e., ulcerative colitis, Crohn's disease); systemic lupus erythematosis; spondyloarthropathies; systemic sclerosis (scleroderma); idiopathic inflammatory myopathies (dermatomyositis, polymyositis); Sjogren's syndrome; systemic vasculitis; sarcoidosis; autoimmune hemolytic anemia (immune pancytopenia, paroxysmal nocturnal hemoglobinuria), autoimmune thrombocytopenia (idiopathic thrombocytopenic purpura, immune-mediated thrombocytopenia); thyroiditis (Grave's disease, Hashimoto's thyroiditis, juvenile lymphocytic thyroiditis, atrophic thyroiditis); diabetes mellitus; immune-mediated renal disease (glomerulonephritis, tubulointerstitial nephritis); demyelinating diseases of the central and peripheral nervous systems such as multiple sclerosis, idiopathic demyelinatingpolyneuropathy or Guillain-Barre syndrome, and chronic inflammatory demyelinating polyneuropathy; hepatobiliary diseases such as infectious hepatitis (hepatitis A, B, C, D, E and other non-hepatotropic viruses), autoimmune chronic active hepatitis, primary biliary cirrhosis, granulomatous hepatitis, and sclerosing cholangitis; inflammatory and fibrotic lung diseases such as cystic fibrosis, gluten-sensitive enteropathy, and Whipple's disease; immunologic diseases of the lung such as eosinophilic pneumonia, idiopathic pulmonary fibrosis and hypersensitivity pneumonitis.

ANTIBODY AND ANTIBODY PRODUCTION

In another aspect, the present invention provides an antibody that binds to the GPR91 and methods for producing such an antibody, including antibodies that function as native GPR91 agonists or antagonists. In one embodiment, the method comprises using isolated epitope-bearing polypeptides of GPR91 or antigenic fragments thereof as an immunogen for producing antibodies that bind to the GPR91 in a known protocol for producing antibodies. In another embodiment, the method comprises using host cells that express recombinant GPR91 as an antigen. In a further embodiment, the method comprises using DNA expression vectors containing the receptor gene to express the receptor as an antigen for producing the antibodies.

Methods for producing antibodies, including polyclonal, monoclonal, monovalent, humanized, human, bispecific, and heteroconjugate antibodies, are well known to skilled artisans.

Methods well known in the art including, but not limited to, in vivo immunization, in vitro immunization, and phage display methods. See, e.g., Sutcliffe et al., supra; Wilson et al., supra, and Bittle et al., J. Gen. Virol., 66:2347-2354 (1985). If in vivo immunization is used, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine residues may be coupled to a carrier using a linker such as maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carriers using a more general linking agent such as glutaraldehyde.

Animals such as rabbits, rats and mice are immunized with either free or carrier-coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 μg of peptide or carrier protein and Freund's adjuvant or any other adjuvant known for stimulating an immune response. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

As one of skill in the art will appreciate, and as discussed above, the polypeptides of the present invention comprising an immunogenic or antigenic epitope can be fused to other polypeptide sequences. For example, the polypeptides of the present invention may be fused with the constant domain of immunoglobulins (IgA, IgE, IgG, IgM), or portions thereof (CH1, CH2, CH3, or any combination thereof and portions thereof) resulting in chimeric polypeptides. Such fusion proteins may facilitate purification and may increase half-life in vivo. This has been shown for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy

or light chains of mammalian immunoglobulins. See, e.g., EP 394,827; Traunecker et al., Nature, 331:84-86 (1988). Enhanced delivery of an antigen across the epithelial barrier to the immune system has been demonstrated for antigens (e.g., insulin) conjugated to an FcRn binding partner such as IgG or Fc fragments (see, e.g., PCT Publications WO 96/22024 and WO 99/04813). IgG Fusion proteins that have a disulfide-linked dimeric structure due to the IgG portion disulfide bonds have also been found to be more efficient in binding and neutralizing other molecules than monomeric polypeptides or fragments thereof alone. See, e.g., Fountoulakis et al., J. Biochem., 270:3958-3964 (1995). Nucleic acids encoding the above epitopes can also be recombined with a gene of interest as an epitope tag (e.g., the hemagglutinin ("HA") tag or flag tag) to aid in detection and purification of the expressed polypeptide. For example, a system described by Janknecht et al. allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht et al., 1991, Proc. Natl. Acad. Sci. USA 88:8972-897). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the open reading frame of the gene is translationally fused to an amino-terminal tag consisting of six histidine residues. The tag serves as a matrix binding domain for the fusion protein. Extracts from cells infected with the recombinant vaccinia virus are loaded onto Ni2+ nitriloacetic acid-agarose column and histidine-tagged proteins can be selectively eluted with imidazole-containing buffers.

Additional fusion proteins of the invention may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffling may be employed to modulate the activities of polypeptides of the invention, such methods can be used to generate polypeptides with altered activity, as well as agonists and antagonists of the polypeptides. See, generally, U.S. Pat. Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten et al., Curr. Opinion Biotechnol. 8:724-33 (1997); Harayama, Trends Biotechnol. 16(2):76-82 (1998); Hansson, et al., J. Mol. Biol. 287:265-76 (1999); and Lorenzo and Blasco, Biotechniques 24(2):308-13 (1998) (each of these patents and publications are hereby incorporated by reference in its entirety).

In one embodiment, alteration of polynucleotides corresponding to SEQ ID NO:1 and the polypeptides encoded by these polynucleotides may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments by homologous or site-specific recombination to generate variation in the polynucleotide sequence. In another embodiment, polynucleotides of the invention, or the encoded polypeptides, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of a polynucleotide encoding a polypeptide of the invention may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

POLYPEPTIDES

In another aspect, the present invention provides an isolated polypeptide having the amino acid sequence selected from the group consisting of SEQ ID NO:2; a variant of SEQ ID NO:2; and a fragment of SEQ ID NO:2. In one embodiment, the isolated polypeptide may be useful as a preventative agent for immunological disorders including arthritis, asthma, immunodeficiency diseases such as AIDS, leukemia, rheumatoid arthritis, granulomatous disease, inflammatory bowel disease, sepsis, acne, neutropenia, neutrophilia, psoriasis, hypersensitivities, such as T-cell mediated cytotoxicity; immune reactions to transplanted organs and tissues, such as host-versus-graft and graft-versus-host diseases, or autoimmunity disorders, such as autoimmune infertility, lense tissue injury, demyelination, systemic lupus erythematosis, drug induced hemolytic anemia, rheumatoid arthritis, Sjogren's disease, and scleroderma. Moreover, the protein may represent a secreted factor that influences the differentiation or behavior of other blood cells, or that recruits hematopoietic cells to sites of injury. Thus, this gene product may be useful in the expansion of stem cells and committed progenitors of various blood lineages, and in the differentiation and/or proliferation of various cell types.

The GPR91 polypeptide may be useful for modulating cytokine production, antigen presentation, or other processes, such as for boosting immune responses, etc. Expression in cells of lymphoid origin, indicates the natural gene product

would be involved in immune functions. Therefore it would also be useful as an agent for immunological disorders including arthritis, asthma, immunodeficiency diseases such as AIDS, leukemia, rheumatoid arthritis, granulomatous disease, inflammatory bowel disease, sepsis, acne, neutropenia, neutrophilia, psoriasis, hypersensitivities, such as T-cell mediated cytotoxicity; immune reactions to transplanted organs and tissues, such as host-versus-graft and graft-versus-host diseases, or autoimmunity disorders, such as autoimmune infertility, lense tissue injury, demyelination, systemic lupus erythematosis, drug induced hemolytic anemia, rheumatoid arthritis, Sjogren's disease, and secleroderma.

Polypeptides of the present invention, including agonists and/or fragments thereof, have uses that include modulating signal transduction activity, in various cells, tissues, and organisms, and particularly in mammalian thymus, small intestine, spinal cord, liver, lung, stomach, uterus, bone marrpow, spleen, lymph node, prostate, brain, kidney, and pancreas tissue, preferably human tissue.

Polypeptides, including agonists, antagonists, and/or fragments thereof, of the present invention have uses which include modulating DNA synthesis; and treating, preventing, and/or ameliorating proliferation disorders.

VECTORS AND HOST CELLS

In another aspect, the present invention provides a vector comprising a nucleotide sequence encoding the GPR91 receptor of the present invention and a host cell comprising such a vector.

By way of example, the host cells may be mammalian cells, (e.g. CHO cells), prokaryotic cells (e.g., E. coli) or yeast cells (e.g., Saccharomyces cerevisiae). A process for producing vertebrate fused polypeptides is further provided and comprises culturing host cells under conditions suitable for expression of vertebrate fused and recovering the same from the cell culture.

RECOMBINANT EXPRESSION FOR GPR91 RECEPTORS

Isolated and purified recombinant GPR91 receptor are provided according to the present invention by incorporating the corresponding nucleotide sequence into expression vectors and expressing the nucleotide sequence in suitable host cells to produce the polypeptide.

EXPRESSION VECTORS

Recombinant expression vectors containing a nucleotide sequence encoding the polypeptide can be prepared using well known techniques. The expression vectors include a nucleotide sequence operably linked to suitable transcriptional or translational regulatory nucleotide sequences such as those derived from mammalian, microbial, viral, or insect genes. Examples of regulatory sequences include transcriptional promoters, operators, enhancers, mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are "operably linked" when the regulatory sequence functionally relates to the nucleotide sequence for the appropriate polypeptide. Thus, a promoter nucleotide sequence is operably linked to a GPR91 receptor sequence if the promoter nucleotide sequence controls the transcription of the appropriate nucleotide sequence.

The ability to replicate in the desired host cells, usually conferred by an origin of replication and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not naturally associated with GPR91 receptor can be incorporated into expression vectors. For example, a nucleotide sequence for a signal peptide (secretory leader) may be fused in-frame to the polypeptide sequence so that the polypeptide is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the appropriate polypeptide. The signal peptide may be cleaved from the polypeptide upon secretion of polypeptide from the cell.

HOST CELLS

Suitable host cells for expression of GPR91 receptor include prokaryotes, yeast, archae, and other eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are well known in the art, e.g., Pouwels et al. Cloning Vectors: A Laboratory Manual, Elsevier, New York (1985). The vector may be a plasmid vector, a single or double-stranded phage vector, or a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The

vectors, in the case of phage and viral vectors also may be and preferably are introduced into cells as packaged or encapsulated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells. Cell-free translation systems could also be employed to produce the protein using RNAs derived from the present DNA constructs.

Prokaryotes useful as host cells in the present invention include gram negative or gram positive organisms such as E. coli or Bacilli. In a prokaryotic host cell, a polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant GPR91 receptor polypeptide. Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase and the lactose promoter system.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. To construct an expression vector using pBR322, an appropriate promoter and a DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden), pGEM1 (Promega Biotec, Madison, Wisconsin., USA), and the pET (Novagen, Madison, Wisconsin, USA) and pRSET (Invitrogen Corporation, Carlsbad, California, USA) series of vectors (Studier, F.W., J. Mol. Biol. 219: 37 (1991); Schoepfer, R. Gene 124: 83 (1993)).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include T7, (Rosenberg, A.H., Lade, B. N., Chui, D-S., Lin, S-W., Dunn, J. J., and Studier, F. W. (1987) Gene (Amst.) 56, 125-135), β-lactamase (penicillinase), lactose promoter system (Chang et al., Nature 275:615,

(1978); and Goeddel et al., Nature 281:544, (1979)), tryptophan (trp) promoter system (Goeddel et al., Nucl. Acids Res. 8:4057, (1980)), and tac promoter (Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, p. 412 (1982)).

Yeasts useful as host cells in the present invention include those from the genus Saccharomyces, Pichia, K. Actinomycetes and Kluyveromyces. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073, (1980)) or other glycolytic enzymes (Holland et al., Biochem. 17:4900, (1978)) such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Fleer et al., Gene, 107:285-195 (1991). Other suitable promoters and vectors for yeast and yeast transformation protocols are well known in the art.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., Proceedings of the National Academy of Sciences USA, 75:1929 (1978). The Hinnen protocol selects for Trp.sup.+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine, and 20 μg/ml uracil.

Mammalian or insect host cell culture systems well known in the art could also be employed to express recombinant GPR91 activating receptors, e.g., Baculovirus systems for production of heterologous proteins in insect cells (Luckow and Summers, Bio/Technology 6:47 (1988)) or Chinese hamster ovary (CHO) cells for mammalian expression may be used. Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and

enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell, e.g., SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication.

Exemplary expression vectors for use in mammalian host cells are well known in the art.GPR91 receptor may, when beneficial, be expressed as a fusion protein that has the GPR91 receptor attached to a fusion segment. The fusion segment often aids in protein purification, e.g., by permitting the fusion protein to be isolated and purified by affinity chromatography. Fusion proteins can be produced by culturing a recombinant cell transformed with a fusion nucleic acid sequence that encodes a protein including the fusion segment attached to either the carboxyl and/or amino terminal end of the protein. Preferred fusion segments include, but are not limited to, glutathione-S-transferase, β-galactosidase, a poly-histidine segment capable of binding to a divalent metal ion, and maltose binding protein.

EXPRESSION AND RECOVERY

According to the present invention, isolated and purified GPR91 receptor may be produced by the recombinant expression systems described above. The method comprises culturing a host cell transformed with an expression vector comprising a nucleotide sequence that encodes the polypeptide under conditions sufficient to promote expression of the polypeptide. The polypeptide is then recovered from culture medium or cell extracts, depending upon the expression system employed. As is known to the skilled artisan, procedures for purifying a recombinant polypeptide will vary according to such factors as the type of host cells employed and whether or not the recombinant polypeptide is secreted into the culture medium. When expression systems that secrete the recombinant polypeptide are employed, the culture medium first may be concentrated. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, e.g., a matrix or substrate having pendant diethylaminoethyl (DEAE)

groups. The matrices can be acrylamide, agarose, dextran, cellulose, or other types commonly employed in protein purification. Also, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Further, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media (e.g., silica gel having pendant methyl or other aliphatic groups), ion exchange-HPLC (e.g., silica gel having pendant DEAE or sulfopropyl (SP) groups), or hydrophobic interaction-HPLC (e.g., silica gel having pendant phenyl, butyl, or other hydrophobic groups) can be employed to further purify the protein. Some or all of the foregoing purification steps, in various combinations, are well known in the art and can be employed to provide an isolated and purified recombinant polypeptide.

Recombinant polypeptide produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification, or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

AGONISTS AND ANTAGONISTS SCREENING

In another aspect, the present invention provides a screening method for identifying GPR91 receptor agonists and antagonists. The screening method comprises exposing a GPR91 receptor to a potential GPR91 agonist/GPR91 antagonist and determining whether the potential agonist/antagonist binds to the receptor. If the potential agonist/antagonist binds to the receptor, there is a strong presumption that the potential agonist/antagonist will actually function as an agonist or antagonist when administered *in vivo* to a patient and exposed to the native GPR91 activating receptor. The GPR91 agonists and GPR91 antagonists identified using the method can be characterized as an agonist or an antagonist by exposing cells capable of producing cytokines to the agonist/antagonist and measuring cytokine production in comparison to non-exposed cells. Agonists will increase cytokine production; antagonists will decrease cytokine production.

Another method for screening comprises transfecting the cells with a reporter gene constructs that contains GPR91 DNA binding sequences. Preferably, the potential agonist/antagonist is an organic compound or polypeptide, including antibodies.

A variety of cell-based functional assays utilize measurements of intracellular calcium concentration to evaluate the activity of proteins in a normal physiological environment. The large and rapid increase in intracellular calcium concentration by stimulation of GRP91 receptors can be detected by intracellular calcium-sensitive probes, including fluorescent dyes, calcium-binding proteins such as the bioluminescent protein, aequorin, and modified green fluorescent protein-calmodulin chimer (Ungrin, M. D., Singh, L. M. R., Stocco, R., Sas, D. E., Abramovitz, M. (1999) An automated aequorin luminescence-based functional calcium assay for G-protein-coupled receptors. Anal. Biochem. 272, 34-42; Takahashi, A., Camacho, P., Lechleiter, J. D., and Herman, B. (1999) Measurement of intracellular calcium. Physiological Reviews 79, 1089-1125; Gonzalez, J. E., Oades, K., Leychis, Y. Harootunian, A., and Negulescu, P. (1999) Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature 388, 882-887; Creton, R., Kreiling, J. A., and Jaffe, L. F. (1999) Calcium imaging with chemiluminescence. Microsc. Res. Tech. 46, 390-397; Prasher, D., McCann, R. O., and Cormier, M. J. (1985) Cloning and expression of the cDNA coding for aequorin, A bioluminescent calcium-binding protein. Biochem. Biophys. Res. Commun. 126, 1259-1268).

Practitioners in the art of screening have used methods to extend the time available to measure fluorescent or luminescent signals. For example, one idea was to add a reagent to alter or slow the kinetics of a cellular reporter enzyme reaction. Examples of this approach are the transformation of flash luciferase-based luminescence to glow-luminescence formats such as the PACKARD LUCLITE.RTM. Luciferase Reporter Gene Assay Kit (see U.S. Pat. No. 5,618,682 and EPO Patent Application 94 102 080.2) or PROMEGA STEADY-GLO.TM. Luciferase Assay System (see U.S. Pat. No. 5,283,179). The screening methods are useful for identifying compounds that may function as drugs for preventing or treating diseases, particularly diseases characterized by relatively low or relatively high cytokine production compared to non-disease states.

High throughput screening methodologies are particularly envisioned for the detection of modulators of GPR91 described herein. Such high throughput screening methods typically involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (e.g., ligand or modulator compounds). Such combinatorial chemical libraries or ligand libraries are then screened in one or more assays to identify those library members (e.g., particular chemical species or subclasses) that display a desired characteristic activity. The compounds so identified can serve as conventional lead compounds, or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical compounds generated either by chemical synthesis or biological synthesis, by combining a number of chemical building blocks (i.e., reagents such as amino acids). As an example, a linear combinatorial library, e.g., a polypeptide or peptide library, is formed by combining a set of chemical building blocks in every possible way for a given compound length (i.e., the number of amino acids in a polypeptide or peptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

The preparation and screening of combinatorial chemical libraries is well known to those having skill in the pertinent art. Combinatorial libraries include, without limitation, peptide libraries (e.g. U.S. Pat. No. 5,010,175; Furka, 1991, Int. J. Pept. Prot. Res., 37:487-493; and Houghton et al., 1991, Nature, 354:84-88). Other chemistries for generating chemical diversity libraries can also be used. Nonlimiting examples of chemical diversity library chemistries include, peptides (PCT Publication No. WO 91/019735), encoded peptides (PCT Publication No. WO 93/20242), random bio-oligomers (PCT Publication No. WO 92/00091), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs et al., 1993, Proc. Natl. Acad. Sci. USA, 90:6909-6913), vinylogous polypeptides (Hagihara et al., 1992, J. Amer. Chem. Soc., 114:6568), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann et al., 1992, J. Amer. Chem. Soc., 114:9217-9218), analogous organic synthesis of small compound libraries (Chen et al., 1994, J. Amer. Chem. Soc., 116:2661), oligocarbamates (Cho et al., 1993, Science, 261:1303), and/or peptidyl phosphonates (Campbell et al., 1994, J. Org. Chem., 59:658), nucleic

acid libraries (see Ausubel, Berger and Sambrook, all supra), peptide nucleic acid libraries (U.S. Pat. No. 5,539,083), antibody libraries (e.g., Vaughn et al., 1996, Nature Biotechnology, 14(3):309-314) and PCT/US96/10287), carbohydrate libraries (e.g., Liang et al., 1996, Science, 274-1520-1522) and U.S. Pat. No. 5,593,853), small organic molecule libraries (e.g., benzodiazepines, Baum C&EN, Jan. 18, 1993, page 33; and U.S. Pat. No. 5,288,514; isoprenoids, U.S. Pat. No. 5,569,588; thiazolidinones and metathiazanones, U.S. Pat. No. 5,549,974; pyrrolidines, U.S. Pat. Nos. 5,525,735 and 5,519,134; morpholino compounds, U.S. Pat. No. 5,506,337; and the like).

Devices for the preparation of combinatorial libraries are commercially available (e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville Ky.; Symphony, Rainin, Woburn, Mass.; 433A Applied Biosystems, Foster City, Calif.; 9050 Plus, Millipore, Bedford, Mass.). In addition, a large number of combinatorial libraries are commercially available (e.g., ComGenex, Princeton, N.J.; Asinex, Moscow, Russia; Tripos, Inc., St. Louis, Mo.; ChemStar, Ltd., Moscow, Russia; 3D Pharmaceuticals, Exton, Pa.; Martek Biosciences, Columbia, Md., and the like).

In one embodiment, the invention provides solid phase based in vitro assays in a high throughput format, where the cell or tissue expressing an ion channel is attached to a solid phase substrate. In such high throughput assays, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to perform a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 96 modulators. If 1536 well plates are used, then a single plate can easily assay from about 100 to about 1500 different compounds. It is possible to assay several different plates per day; thus, for example, assay screens for up to about 6,000-20,000 different compounds are possible using the described integrated systems.

In another of its aspects, the present invention encompasses screening and small molecule (e.g., drug) detection assays which involve the detection or identification of small molecules that can bind to a given protein, i.e., a GPR91 polypeptide or peptide. Particularly preferred are assays suitable for high throughput screening methodologies.

In such binding-based detection, identification, or screening assays, a functional assay is not typically required. All that is needed is a target protein, preferably substantially purified, and a library or panel of compounds (e.g., ligands, drugs, small molecules) or biological entities to be screened or assayed for binding to the protein target. Preferably, most small molecules that bind to the target protein will modulate activity in some manner, due to preferential, higher affinity binding to functional areas or sites on the protein.

An example of such an assay is the fluorescence based thermal shift assay (3-Dimensional Pharmaceuticals, Inc., 3DP, Exton, Pa.) as described in U.S. Pat. Nos. 6,020,141 and 6,036,920 to Pantoliano et al.; see also, J. Zimmerman, 2000, Gen. Eng. News, 20(8)). The assay allows the detection of small molecules (e.g., drugs, st ligands) that bind to expressed, and preferably purified, ion channel polypeptide based on affinity of binding determinations by analyzing thermal unfolding curves of protein-drug or ligand complexes. The drugs or binding molecules determined by this technique can be further assayed, if desired, by methods, such as those described herein, to determine if the molecules affect or modulate function or activity of the target protein.

To purify a GPR91 polypeptide or peptide to measure a biological binding or ligand binding activity, the source may be a whole cell lysate that can be prepared by successive freeze-thaw cycles (e.g., one to three) in the presence of standard protease inhibitors. The GPR91 polypeptide may be partially or completely purified by standard protein purification methods, e.g., affinity chromatography using specific antibody described infra, or by ligands specific for an epitope tag engineered into the recombinant GPR91 polypeptide molecule, also as described herein. Binding activity can then be measured as described.

Compounds which are identified according to the methods provided herein, and which modulate or regulate the biological activity or physiology of the GPR91 polypeptides according to the present invention are a preferred embodiment of this invention. It is contemplated that such modulatory compounds may be employed in treatment and therapeutic methods for treating a condition that is mediated by GPR91 polypeptides by administering to an individual in need of such treatment a therapeutically effective amount of the compound identified by the methods described herein.

In addition, the present invention provides methods for treating an individual in need of such treatment for a disease, disorder, or condition that is mediated by the GPR91 polypeptides of the invention, comprising administering to the individual a therapeutically effective amount of the GPR91-modulating compound identified by a method provided herein.

ANTIBODIES

Further polypeptides of the invention relate to antibodies and T-cell antigen receptors (TCR) which immunospecifically bind a polypeptide, polypeptide fragment, or variant of SEQ ID NO:2, and/or an epitope, of the present invention (as determined by immunoassays well known in the art for assaying specific antibody-antigen binding). Antibodies of the invention include, but are not limited to, polyclonal, monoclonal, monovalent, bispecific, heteroconjugate, multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above.

The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. The immunoglobulin molecules of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Moreover, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules, as well as, antibody fragments (such as, for example, Fab and F(ab')2 fragments) which are capable of specifically binding to protein. Fab and F(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation of the animal or plant, and may have less non-specific tissue binding than an intact antibody (Wahl et al., J. Nucl. Med. 24:316-325 (1983)). Thus, these fragments are preferred, as well as the products of a FAB or other immunoglobulin expression library. Moreover, antibodies of the present invention include chimeric, single chain, and humanized antibodies.

The antibodies may be human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')2, Fd,

single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains. The antibodies of the invention may be from any animal origin including birds and mammals. Preferably, the antibodies are human, murine (e.g., mouse and rat), donkey, ship rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulin and that do not express endogenous immunoglobulins, as described infra and, for example in, U.S. Pat. No. 5,939,598 by Kucherlapati et al.

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of a polypeptide of the present invention or may be specific for both a polypeptide of the present invention as well as for a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, et al., J. Immunol. 147:60-69 (1991); U.S. Pat. Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny et al., J. Immunol. 148:1547-1553 (1992).

Antibodies of the present invention may be described or specified in terms of the epitope(s) or portion(s) of a polypeptide of the present invention which they recognize or specifically bind. The epitope(s) or polypeptide portion(s) may be specified as described herein, e.g., by N-terminal and C-terminal positions, by size in contiguous amino acid residues, or listed in the Tables and Figures. Antibodies which specifically bind any epitope or polypeptide of the present invention may also be excluded. Therefore, the present invention includes antibodies that specifically bind polypeptides of the present invention, and allows for the exclusion of the same.

Antibodies of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies that do not bind any other analog, ortholog, or homologue of a polypeptide of the present invention are included. Antibodies that bind polypeptides with at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 65%, at least 60%, at least 55%, and at least 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention.

In specific embodiments, antibodies of the present invention cross-react with murine, rat and/or rabbit homologues of human proteins and the corresponding epitopes thereof. Antibodies that do not bind polypeptides with less than 95%, less than 90%, less than 85%, less than 80%, less than 75%, less than 70%, less than 65%, less than 60%, less than 55%, and less than 50% identity (as calculated using methods known in the art and described herein) to a polypeptide of the present invention are also included in the present invention. In a specific embodiment, the above-described cross-reactivity is with respect to any single specific antigenic or immunogenic polypeptide, or combination(s) of 2, 3, 4, 5, or more of the specific antigenic and/or immunogenic polypeptides disclosed herein. Further included in the present invention are antibodies which bind polypeptides encoded by polynucleotides which hybridize to a polynucleotide of the present invention under stringent hybridization conditions (as described herein). Antibodies of the present invention may also be described or specified in terms of their binding affinity to a polypeptide of the invention. Preferred binding affinities include those with a dissociation constant or Kd less than 5.times.10-2 M, 10-2 M, 5.times.10-3 M, 10-3 M, 5.times.10-4 M, 10-4 M, 5.times.10-5 M, 10-5 M, 5.times.10-6 M, 10-6M, 5.times.10-7 M, 107 M, 5.times.10-8 M, 10-8 M, 5.times.10-9 M, 10-9 M, 5.times.10-10 M, 10-10 M, 5.times.10-11 M, 10-11 M, 5.times.10-12 M, 10-12 M, 5.times.10-13 M, 10-13 M, 5.times.10-14 M, 10-14 M, 5.times.10-15 M, or 10-15 M.

The invention also provides antibodies that competitively inhibit binding of an antibody to an epitope of the invention as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the antibody competitively inhibits

binding to the epitope by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50%.

Antibodies of the present invention may act as agonists or antagonists of the polypeptides of the present invention. For example, the present invention includes antibodies which disrupt the receptor/ligand interactions with the polypeptides of the invention either partially or fully. Preferably, antibodies of the present invention bind an antigenic epitope disclosed herein, or a portion thereof. The invention features both receptor-specific antibodies and ligand-specific antibodies. The invention also features receptor-specific antibodies which do not prevent ligand binding but prevent receptor activation. Receptor activation (i.e., signaling) may be determined by techniques described herein or otherwise known in the art. For example, receptor activation can be determined by detecting the phosphorylation (e.g., tyrosine or serine/threonine) of the receptor or its substrate by immunoprecipitation followed by western blot analysis (for example, as described supra). In specific embodiments, antibodies are provided that inhibit ligand activity or receptor activity by at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, or at least 50% of the activity in absence of the antibody.

The invention also features receptor-specific antibodies which both prevent ligand binding and receptor activation as well as antibodies that recognize the receptor-ligand complex, and, preferably, do not specifically recognize the unbound receptor or the unbound ligand. Likewise, included in the invention are neutralizing antibodies which bind the ligand and prevent binding of the ligand to the receptor, as well as antibodies which bind the ligand, thereby preventing receptor activation, but do not prevent the ligand from binding the receptor. Further included in the invention are antibodies which activate the receptor. These antibodies may act as receptor agonists, i.e., potentiate or activate either all or a subset of the biological activities of the ligand-mediated receptor activation, for example, by inducing dimerization of the receptor. The antibodies may be specified as agonists, antagonists or inverse agonists for biological activities comprising the specific biological activities of the peptides of the invention disclosed herein. The above antibody agonists can be made using methods known in the art. See, e.g., PCT publication WO 96/40281; U.S. Pat. No.

5,811,097; Deng et al., Blood 92(6):1981-1988 (1998); Chen et al., Cancer Res. 58(16):3668-3678 (1998); Harrop et al., J. Immunol. 161(4):1786-1794 (1998); Zhu et al., Cancer Res. 58(15):3209-3214 (1998); Yoon et al., J. Immunol. 160(7):3170-3179 (1998); Prat et al., J. Cell. Sci. 11 (Pt2):237-247 (1998); Pitard et al., J. Immunol. Methods 205(2):177-190 (1997); Liautard et al., Cytokine 9(4):233-241 (1997); Carlson et al., J. Biol. Chem . . . 272(17):11295-11301 (1997); Taryman et al., Neuron 14(4):755-762 (1995); Muller et al., Structure 6(9):1153-1167 (1998); Bartunek et a Cytokine 8(1):14-20 (1996) (which are all incorporated by reference herein in their entireties).

Antibodies of the present invention may be used, for example, but not limited to, to purify, detect, and target the polypeptides of the present invention, including both in vitro and in vivo diagnostic and therapeutic methods. For example, the antibodies have use in immunoassays for qualitatively and quantitatively measuring levels of the polypeptides of the present invention in biological samples. See, e.g., Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988) (incorporated by reference herein in its entirety).

As discussed in more detail below, the antibodies of the present invention may be used either alone or in combination with other compositions. The antibodies may further be recombinantly fused to a heterologous polypeptide at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to polypeptides or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as labels in detection assays and effector molecules such as heterologous polypeptides, drugs, radionucleotides, or toxins. See, e.g., PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Pat. No. 5,314,995; and EP 396,387.

The antibodies of the invention include derivatives that are modified, i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from generating an anti-idiotypic response. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known

protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

The antibodies of the present invention may be generated by any suitable method known in the art. The antibodies of the present invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan (Harlow, et al., Antibodies: A Laboratory Manual, (Cold spring Harbor Laboratory Press, 2.sup.nd ed. (1988), which is hereby incorporated herein by reference in its entirety). For example, a polypeptide of the invention can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for the antigen. The administration of the polypeptides of the present invention may entail one or more injections of an immunizing agent and, if desired, an adjuvant. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Such adjuvants are also well known in the art. For the purposes of the invention, "immunizing agent" may be defined as a polypeptide of the invention, including fragments, variants, and/or derivatives thereof, in addition to fusions with heterologous polypeptides and other forms of the polypeptides described herein.

Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections, though they may also be given intramuscularly, and/or through IV). The immunizing agent may include polypeptides of the present invention or a fusion protein or variants thereof. Depending upon the nature of the polypeptides (i.e., percent hydrophobicity, percent hydrophilicity, stability, net charge, isoelectric point etc.), it may be useful to conjugate the immunizing agent to a protein known to be

immunogenic in the mammal being immunized. Such conjugation includes either chemical conjugation by derivitizing active chemical functional groups to both the polypeptide of the present invention and the immunogenic protein such that a covalent bond is formed, or through fusion-protein based methodology, or other methods known to the skilled artisan. Examples of such immunogenic proteins include, but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Additional examples of adjuvants which may be employed includes the MPL-TDM adjuvant (monophosphoryl lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

The antibodies of the present invention may comprise monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975) and U.S. Pat. No. 4,376,110, by Harlow, et al., Antibodies: A Laboratory Manual, (Cold spring Harbor Laboratory Press, 2.sup.nd ed. (1988), by Hammerling, et al., Monoclonal Antibodies and T-Cell Hybridomas (Elsevier, N.Y., (1981)), or other methods known to the artisan. Other examples of methods which may be employed for producing monoclonal antibodies includes, but are not limited to, the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In a hybridoma method, a mouse, a humanized mouse, a mouse with a human immune system, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro.

The immunizing agent will typically include polypeptides of the present invention or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986), pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif. and the American Type Culture Collection, Manassas, Va. As inferred throughout the specification, human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63).

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against the

polypeptides of the present invention. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoadsorbant assay (ELISA). Such techniques are known in the art and within the skill of the artisan. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollart, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, supra). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640. Alternatively, the hybridoma cells may be grown in vivo as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-sepharose, hydroxyapatite chromatography, gel exclusion chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The skilled artisan would acknowledge that a variety of methods exist in the art for the production of monoclonal antibodies and thus, the invention is not limited to their sole production in hydridomas. For example, the monoclonal antibodies may be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567. In this context, the term "monoclonal antibody" refers to an antibody derived from a single eukaryotic, phage, or prokaryotic clone. The DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies, or such chains from human, humanized, or other sources). The hydridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transformed into host cells such as Simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by

substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison et al, supra) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art and are discussed in detail in the Examples herein. In a non-limiting example, mice can be immunized

with a polypeptide of the invention or a cell expressing such peptide. Once an immune response is detected, e.g., antibodies specific for the antigen are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well-known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with an antigen of the invention with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain.

For example, the antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage

with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., J. Immunol. Methods 182:41-50 (1995); Ames et al., J. Immunol. Methods 184:177-186 (1995); Kettleborough et al., Eur. J. Immunol. 24:952-958 (1994); Persic et al., Gene 187 9-18 (1997); Burton et al., Advances in Immunology 57:191-280 (1994); PCT application No. PCT/GB91/01134; PCT publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Pat. Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax et al., BioTechniques 12(6):864-869 (1992); and Sawai et al., AJRI 34:26-34 (1995); and Better et al., Science 240:1041-1043 (1988) (said references incorporated by reference in their entireties). Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Pat. Nos. 4,946,778 and 5,258,498; Huston et al., Methods in Enzymology 203:46-88 (1991); Shu et al., PNAS 90:7995-7999 (1993); and Skerra et al., Science 240:1038-1040 (1988).

For some uses, including in vivo use of antibodies in humans and in vitro detection assays, it may be preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, Science 229:1202 (1985); Oi et al.,

BioTechniques 4:214 (1986); Gillies et al., (1989) J. Immunol. Methods 125:191-202; U.S. Pat. Nos. 5,807,715; 4,816,567; and 4,816397, which are incorporated herein by reference in their entirety. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and a framework regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Pat. No. 5,585,089; Riechmann et al., Nature 332:323 (1988), which are incorporated herein by reference in their entireties.). Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Molecular Immunology 28(4/5):489-498 (1991); Studnicka et al., Protein Engineering 7(6):805-814 (1994); Roguska. et al., PNAS 91:969-973 (1994)), and chain shuffling (U.S. Pat. No. 5,565,332). Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the methods of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some

CDR residues and possible some FR residues are substituted from analogous sites in rodent antibodies.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988)1 and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety. The techniques of cole et al., and Boerder et al., are also available for the preparation of human monoclonal antibodies (cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Riss, (1985); and Boerner et al., J. Immunol., 147(1):86-95, (1991)).

Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified

embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., all or a portion of a polypeptide of the invention. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, Int. Rev. Immunol. 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.), Genpharm (San Jose, Calif.), and Medarex, Inc. (Princeton, N.J.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Similarly, human antibodies can be made by introducing human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and creation of an antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,106, and in the following scientific publications: Marks et al., Biotechnol., 10:779-783 (1992); Lonberg et al., Nature 368:856-859 (1994); Fishwild et al., Nature Biotechnol., 14:845-51 (1996); Neuberger, Nature Biotechnol., 14:826 (1996); Lonberg and Huszer, Intern. Rev. Immunol., 13:65-93 (1995).

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, e.g., a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers et al., Bio/technology 12:899-903 (1988)).

Further, antibodies to the polypeptides of the invention can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" polypeptides of the invention using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, FASEB J. 7(5):437-444; (1989) and Nissinoff, J. Immunol. 147(8):2429-2438 (1991)). For example, antibodies which bind to and competitively inhibit polypeptide multimerization and/or binding of a polypeptide of the invention to a ligand can be used to generate anti-idiotypes that "mimic" the polypeptide multimerization and/or binding domain and, as a consequence, bind to and neutralize polypeptide and/or its ligand. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize polypeptide ligand. For example, such anti-idiotypic antibodies can be used to bind a polypeptide of the invention and/or to bind its ligands/receptors, and thereby block its biological activity.

The antibodies of the present invention may be bispecific antibodies. Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present invention, one of the binding specificities may be directed towards a polypeptide of the present invention, the other may be for any other antigen, and preferably for a cell-surface protein, receptor, receptor subunit, tissue-specific antigen, virally derived protein, virally encoded envelope protein, bacterially derived protein, or bacterial surface protein, etc.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature, 305:537-539 (1983). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The

purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published May 13, 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transformed into a suitable host organism. For further details of generating bispecific antibodies see, for example Suresh et al., Meth. In Enzym., 121:210 (1986).

Heteroconjugate antibodies are also contemplated by the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for the treatment of HIV infection (WO 91/00360; WO 92/20373; and EP03089). It is contemplated that the antibodies may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioester bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

POLYNUCLEOTIDES ENCODING ANTIBODIES

The invention further provides polynucleotides comprising a nucleotide sequence encoding an antibody of the invention and fragments thereof. The invention also encompasses polynucleotides that hybridize under stringent or lower stringency hybridization conditions, e.g., as defined supra, to polynucleotides that encode an antibody, preferably, that specifically binds to a polypeptide of the invention, preferably, an antibody that binds to a polypeptide having the amino acid sequence of SEQ ID NO:2.

The polynucleotides may be obtained, and the nucleotide sequence of the polynucleotides determined, by any method known in the art. For example, if the nucleotide sequence of the antibody is known, a polynucleotide encoding the antibody may be assembled from chemically synthesized oligonucleotides (e.g., as described in Kutmeier et al., BioTechniques 17:242 (1994)), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the antibody, annealing and ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

Alternatively, a polynucleotide encoding an antibody may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular antibody is not available, but the sequence of the antibody molecule is known, a nucleic acid encoding the immunoglobulin may be chemically synthesized or obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the antibody, such as hybridoma cells selected to express an antibody of the invention) by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

Once the nucleotide sequence and corresponding amino acid sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, for example, the techniques described in Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. and Ausubel et al., eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties), to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

In a specific embodiment, the amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the sequences of the complementarity determining regions (CDRs) by methods that are well know in the art, e.g., by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, e.g., into human framework regions to humanize a non-human antibody, as described supra. The framework regions may be naturally occurring or consensus framework regions, and preferably human framework regions (see, e.g., Chothia et al., J. Mol. Biol. 278: 457-479 (1998) for a listing of human framework regions). Preferably, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds a polypeptide of the invention. Preferably, as discussed supra, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., Proc. Natl. Acad. Sci. 81:851-855 (1984); Neuberger et al., Nature 312:604-608 (1984); Takeda et al., Nature 314:452-454 (1985)) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described supra, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region, e.g., humanized antibodies.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, Science 242:423-42 (1988); Huston et al., Proc. Natl. Acad. Sci. USA 85:5879-5883 (1988); and Ward et al., Nature

334:544-54 (1989)) can be adapted to produce single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Techniques for the assembly of functional Fv fragments in E. coli may also be used (Skerra et al., Science242:1038-1041 (1988)).

METHODS OF PRODUCING ANTIBODIES

The antibodies of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

Recombinant expression of an antibody of the invention, or fragment, derivative or analog thereof, (e.g., a heavy or light chain of an antibody of the invention or a single chain antibody of the invention), requires construction of an expression vector containing a polynucleotide that encodes the antibody. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or portion thereof (preferably containing the heavy or light chain variable domain), of the invention has been obtained, the vector for the production of the antibody molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody encoding nucleotide sequence are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing antibody coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an antibody molecule of the invention, or a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Pat. No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques

to produce an antibody of the invention. Thus, the invention includes host cells containing a polynucleotide encoding an antibody of the invention, or a heavy or light chain thereof, or a single chain antibody of the invention, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the antibody molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (e.g., E. coli, B. subtilis) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (e.g., Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as Escherichia coli, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecule, are used for the expression of a recombinant antibody molecule. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system

for antibodies (Foecking et al., Gene 45:101 (1986); Cockett et al., Bio/Technology 8:2 (1990)).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody molecule, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the E. coli expression vector pUR278 (Ruther et al., EMBO J. 2:1791 (1983)), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:3101-3109 (1985); Van Heeke & Schuster, J. Biol. Chem. . . . 24:5503-5509 (1989)); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in Spodoptera frugiperda cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is

viable and capable of expressing the antibody molecule in infected hosts. (e.g., see Logan & Shenk, Proc. Natl. Acad. Sci. USA 81:355-359 (1984)). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., Methods in Enzymol. 153:51-544 (1987)).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERY, BHK, Hela, COS, MDCK, 293, 3T3, WI38, and in particular, breast cancer cell lines such as, for example, BT483, Hs578T, HTB2, BT20 and T47D, and normal mammary gland cell line such as, for example, CRL7030 and Hs578Bst.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the antibody molecule may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective

media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the antibody molecule. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody molecule.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223 (1977)), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA 48:202 (1992)), and adenine phosphoribosyltransferase (Lowy et al., Cell 22:817 (1980)) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler et al., Natl. Acad. Sci. USA 77:357 (1980); O'Hare et al., Proc. Natl. Acad. Sci. USA 78:1527 (1981)); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA 78:2072 (1981)); neo, which confers resistance to the aminoglycoside G-418 Clinical Pharmacy 12:488-505; Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62:191-217 (1993); May, 1993, TIB TECH 11(5):155-215); and hygro, which confers resistance to hygromycin (Santerre et al., Gene 30:147 (1984)). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli et al. (eds), Current Protocols in Human Genetics, John Wiley & Sons, NY (1994); Colberre-Garapin et al., J. Mol. Biol. 150:1 (1981), which are incorporated by reference herein in their entireties.

The expression levels of an antibody molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian

cells in DNA cloning, Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody will also increase (Crouse et al., Mol. Cell. Biol. 3:257 (1983)).

The host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, Nature 322:52 (1986); Kohler, Proc. Natl. Acad. Sci. USA 77:2197 (1980)). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once an antibody molecule of the invention has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method known in the art for purification of an immunoglobulin molecule, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. In addition, the antibodies of the present invention or fragments thereof can be fused to heterologous polypeptide sequences described herein or otherwise known in the art, to facilitate purification.

The present invention encompasses antibodies recombinantly fused or chemically conjugated (including both covalently and non-covalently conjugations) to a polypeptide (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. The antibodies may be specific for antigens other than polypeptides (or portion thereof, preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 amino acids of the polypeptide) of the present invention. For example,

antibodies may be used to target the polypeptides of the present invention to particular cell types, either in vitro or in vivo, by fusing or conjugating the polypeptides of the present invention to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to the polypeptides of the present invention may also be used in vitro immunoassays and purification methods using methods known in the art. See e.g., Harbor et al., supra, and PCT publication WO 93/21232; EP 439,095; Naramura et al., Immunol. Lett. 39:91-99 (1994); U.S. Pat. No. 5,474,981; Gillies et al., PNAS 89:1428-1432 (1992); Fell et al., J. Immunol. 146:2446-2452(1991), which are incorporated by reference in their entireties.

The present invention further includes compositions comprising the polypeptides of the present invention fused or conjugated to antibody domains other than the variable regions. For example, the polypeptides of the present invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a polypeptide of the present invention may comprise the constant region, hinge region, CH1 domain, CH2 domain, and CH3 domain or any combination of whole domains or portions thereof. The polypeptides may also be fused or conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the polypeptides of the present invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the polypeptides to portions of IgA and IgM. Methods for fusing or conjugating the polypeptides of the present invention to antibody portions are known in the art. See, e.g., U.S. Pat. Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 91/06570; Ashkenazi et al., Proc. Natl. Acad. Sci. USA 88:10535-10539 (1991); Zheng et al., J. Immunol. 154:5590-5600 (1995); and Vil et al., Proc. Natl. Acad. Sci. USA 89:11337-11341(1992) (said references incorporated by reference in their entireties).

As discussed, supra, the polypeptides corresponding to a polypeptide, polypeptide fragment, or a variant of SEQ ID NO:2 may be fused or conjugated to the above antibody portions to increase the in vivo half life of the polypeptides or for use in immunoassays using methods known in the art. Further, the polypeptides corresponding to SEQ ID NO:2 may be fused or conjugated to the

above antibody portions to facilitate purification. One reported example describes chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EP 394,827; Traunecker et al., Nature 331:84-86 (1988). The polypeptides of the present invention fused or conjugated to an antibody having disulfide-linked dimeric structures (due to the IgG) may also be more efficient in binding and neutralizing other molecules, than the monomeric secreted protein or protein fragment alone. (Fountoulakis et al., J. Biochem. 270:3958-3964 (1995)). In many cases, the Fc part in a fusion protein is beneficial in therapy and diagnosis, and thus can result in, for example, improved pharmacokinetic properties. (EP A 232,262). Alternatively, deleting the Fc part after the fusion protein has been expressed, detected, and purified, would be desired. For example, the Fc portion may hinder therapy and diagnosis if the fusion protein is used as an antigen for immunizations. In drug discovery, for example, human proteins, such as hIL-5, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. (See, Bennett et al., J. Molecular Recognition 8:52-58 (1995); Johanson et al., J. Biol. Chem. . . . 270:9459-9471 (1995).

Moreover, the antibodies or fragments thereof of the present invention can be fused to marker sequences, such as a peptide to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif., 91311), among others, many of which are commercially available. As described in Gentz et al., Proc. Natl. Acad. Sci. USA 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., Cell 37:767 (1984)) and the "flag" tag.

The present invention further encompasses antibodies or fragments thereof conjugated to a diagnostic or therapeutic agent. The antibodies can be used diagnostically to, for example, monitor the development or progression of a tumor as part of a clinical testing procedure to, e.g., determine the efficacy of a given

treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive materials, positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions. The detectable substance may be coupled or conjugated either directly to the antibody (or fragment thereof) or indirectly, through an intermediate (such as, for example, a linker known in the art) using techniques known in the art. See, for example, U.S. Pat. No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics according to the present invention. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include 125I, 131I, 111In or 99Tc.

Further, an antibody or fragment thereof may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters such as, for example, 213Bi. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologues thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g.,

daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

The conjugates of the invention can be used for modifying a given biological response, the therapeutic agent or drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, a-interferon, .beta.-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF-alpha, TNF-beta, AIM I (See, International Publication No. WO 97/33899), AIM II (See, International Publication No. WO 97/34911), Fas Ligand (Takahashi et al., Int. Immunol., 6:1567-1574 (1994)), VEGI (See, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

Techniques for conjugating such therapeutic moiety to antibodies are well known, see, e.g., Amon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer

Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., "The Preparation And Cytotoxic Properties Of Antibody-Toxin Conjugates", Immunol. Rev. 62:119-58 (1982).

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Pat. No. 4,676,980, which is incorporated herein by reference in its entirety.

An antibody, with or without a therapeutic moiety conjugated to it, administered alone or in combination with cytotoxic factor(s) and/or cytokine(s) can be used as a therapeutic.

The present invention also encompasses the creation of synthetic antibodies directed against the polypeptides of the present invention. One example of synthetic antibodies is described in Radrizzani, M., et al., Medicina, (Aires), 59(6):753-8, (1999)). Recently, a new class of synthetic antibodies has been described and are referred to as molecularly imprinted polymers (MIPs) (Semorex, Inc.). Antibodies, peptides, and enzymes are often used as molecular recognition elements in chemical and biological sensors. However, their lack of stability and signal transduction mechanisms limits their use as sensing devices. Molecularly imprinted polymers (MIPs) are capable of mimicking the function of biological receptors but with less stability constraints. Such polymers provide high sensitivity and selectivity while maintaining excellent thermal and mechanical stability. MIPs have the ability to bind to small molecules and to target molecules such as organics and proteins' with equal or greater potency than that of natural antibodies. These "super" MIPs have higher affinities for their target and thus require lower concentrations for efficacious binding.

During synthesis, the MIPs are imprinted so as to have complementary size, shape, charge and functional groups of the selected target by using the target molecule itself (such as a polypeptide, antibody, etc.), or a substance having a very similar structure, as its "print" or "template." MIPs can be derivatized with the same reagents afforded to antibodies. For example, fluorescent `super` MIPs can be coated onto beads or wells for use in highly sensitive separations or assays, or for use in high throughput screening of proteins.

Moreover, MIPs based upon the structure of the polypeptide(s) of the present invention may be useful in screening for compounds that bind to the

polypeptide(s) of the invention. Such a MIP would serve the role of a synthetic "receptor" by minimicking the native architecture of the polypeptide. In fact, the ability of a MIP to serve the role of a synthetic receptor has already been demonstrated for the estrogen receptor (Ye, L., Yu, Y., Mosbach, K, Analyst., 126(6):760-5, (2001); Dickert, F, L., Hayden, O., Halikias, K, P, Analyst., 126(6):766-71, (2001)). A synthetic receptor may either be mimicked in its entirety (e.g., as the entire protein), or mimicked as a series of short peptides corresponding to the protein (Rachkov, A., Minoura, N, Biochim, Biophys, Acta., 1544(1-2):255-66, (2001)). Such a synthetic receptor MIPs may be employed in any one or more of the screening methods described elsewhere herein.

MIPs have also been shown to be useful in "sensing" the presence of its mimicked molecule (Cheng, Z., Wang, E., Yang, X, Biosens, Bioelectron., 16(3):179-85, (2001); Jenkins, A, L., Yin, R., Jensen, J. L, Analyst., 126(6):798-802, (2001); Jenkins, A, L., Yin, R., Jensen, J. L, Analyst., 126(6):798-802, (2001)). For example, a MIP designed using a polypeptide of the present invention may be used in assays designed to identify, and potentially quantitate, the level of said polypeptide in a sample. Such a MIP may be used as a substitute for any component described in the assays, or kits, provided herein (e.g., ELISA, etc.).

A number of methods may be employed to create MIPs to a specific receptor, ligand, polypeptide, peptide, organic molecule. Several preferred methods are described by Esteban et al in J. Anal, Chem., 370(7):795-802, (2001), which is hereby incorporated herein by reference in its entirety in addition to any references cited therein. Additional methods are known in the art and are encompassed by the present invention, such as for example, Hart, B, R., Shea, K, J. J. Am. Chem, Soc., 123(9):2072-3, (2001); and Quaglia, M., Chenon, K., Hall, A, J., De, Lorenzi, E., Sellergren, B, J. Am. Chem, Soc., 123(10):2146-54, (2001); which are hereby incorporated by reference in their entirety herein.

USES FOR ANTIBODIES DIRECTED AGAINST POLYPEPTIDES OF THE INVENTION

The antibodies of the present invention have various utilities. For example, such antibodies may be used in diagnostic assays to detect the presence or quantification of the polypeptides of the invention in a sample. Such a diagnostic assay may be comprised of at least two steps. The first, subjecting a sample with

the antibody, wherein the sample is a tissue (e.g., human, animal, etc.), biological fluid (e.g., blood, urine, sputum, semen, amniotic fluid, saliva, etc.), biological extract (e.g., tissue or cellular homogenate, etc.), a protein microchip (e.g., See Arenkov P, et al., Anal Biochem., 278(2):123-131 (2000)), or a chromatography column, etc. And a second step involving the quantification of antibody bound to the substrate. Alternatively, the method may additionally involve a first step of attaching the antibody, either covalently, electrostatically, or reversibly, to a solid support, and a second step of subjecting the bound antibody to the sample, as defined above and elsewhere herein.

Various diagnostic assay techniques are known in the art, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogenous phases (Zola, Monoclonal antibodies: A Manual of Techniques, CRC Press, Inc., (1987), pp147-158). The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as 2H, 14C, 32P, or 125I, a florescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase, green fluorescent protein, or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); Dafvid et al., Biochem., 13:1014 (1974); Pain et al., J. Immunol. Metho., 40:219(1981); and Nygren, J. Histochem. And Cytochem., 30:407 (1982).

Antibodies directed against the polypeptides of the present invention are useful for the affinity purification of such polypeptides from recombinant cell culture or natural sources. In this process, the antibodies against a particular polypeptide are immobilized on a suitable support, such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the polypeptides to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except for the desired polypeptides, which are bound to the

immobilized antibody. Finally, the support is washed with another suitable solvent that will release the desired polypeptide from the antibody.

THERAPEUTIC USES OF ANTIBODIES

The present invention is further directed to antibody-based therapies which involve administering antibodies of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating one or more of the disclosed diseases, disorders, or conditions. Therapeutic compounds of the invention include, but are not limited to, antibodies of the invention (including fragments, analogs and derivatives thereof as described herein) and nucleic acids encoding antibodies of the invention (including fragments, analogs and derivatives thereof and anti-idiotypic antibodies as described herein). The antibodies of the invention can be used to treat, inhibit or prevent diseases, disorders or conditions associated with aberrant expression and/or activity of a polypeptide of the invention, including, but not limited to, any one or more of the diseases, disorders, or conditions described herein. The treatment and/or prevention of diseases, disorders, or conditions associated with aberrant expression and/or activity of a polypeptide of the invention includes, but is not limited to, alleviating symptoms associated with those diseases, disorders or conditions. Antibodies of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

A summary of the ways in which the antibodies of the present invention may be used therapeutically includes binding polynucleotides or polypeptides of the present invention locally or systemically in the body or by direct cytotoxicity of the antibody, e.g. as mediated by complement (CDC) or by effector cells (ADCC). Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the antibodies of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

The antibodies of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic growth factors (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

The antibodies of the invention may be administered alone or in combination with other types of treatments (e.g., radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents). Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, human antibodies, fragments derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

It is preferred to use high affinity and/or potent in vivo inhibiting and/or neutralizing antibodies against polypeptides or polynucleotides of the present invention, fragments or regions thereof, for both immunoassays directed to and therapy of disorders related to polynucleotides or polypeptides, including fragments thereof, of the present invention. Such antibodies, fragments, or regions, will preferably have an affinity for polynucleotides or polypeptides of the invention, including fragments thereof.

Antibodies directed against polypeptides of the present invention are useful for inhibiting allergic reactions in animals. For example, by administering a therapeutically acceptable dose of an antibody, or antibodies, of the present invention, or a cocktail of the present antibodies, or in combination with other antibodies of varying sources, the animal may not elicit an allergic response to antigens.

Likewise, one could envision cloning the gene encoding an antibody directed against a polypeptide of the present invention, said polypeptide having the potential to elicit an allergic and/or immune response in an organism, and transforming the organism with said antibody gene such that it is expressed (e.g., constitutively, inducibly, etc.) in the organism. Thus, the organism would effectively become resistant to an allergic response resulting from the ingestion or presence of such an immune/allergic reactive polypeptide. Moreover, such a use of the antibodies of the present invention may have particular utility in preventing and/or ameliorating autoimmune diseases and/or disorders, as such conditions are typically a result of antibodies being directed against endogenous proteins. For example, in the instance where the polypeptide of the present invention is responsible for modulating the immune response to auto-antigens, transforming the organism and/or individual with a construct comprising any of the promoters

disclosed herein or otherwise known in the art, in addition, to a polynucleotide encoding the antibody directed against the polypeptide of the present invention could effective inhibit the organisms immune system from eliciting an immune response to the auto-antigen(s). Detailed descriptions of therapeutic and/or gene therapy applications of the present invention are provided elsewhere herein.

Alternatively, antibodies of the present invention could be produced in a plant (e.g., cloning the gene of the antibody directed against a polypeptide of the present invention, and transforming a plant with a suitable vector comprising said gene for constitutive expression of the antibody within the plant), and the plant subsequently ingested by an animal, thereby conferring temporary immunity to the animal for the specific antigen the antibody is directed towards (See, for example, U.S. Pat. Nos. 5,914,123 and 6,034,298).

In another embodiment, antibodies of the present invention, preferably polyclonal antibodies, more preferably monoclonal antibodies, and most preferably single-chain antibodies, can be used as a means of inhibiting gene expression of a particular gene, or genes, in a human, mammal, and/or other organism. See, for example, International Publication Number WO 00/05391, published Feb. 3, 2000, to Dow Agrosciences LLC. The application of such methods for the antibodies of the present invention are known in the art, and are more particularly described elsewhere herein.

In yet another embodiment, antibodies of the present invention may be useful for multimerizing the polypeptides of the present invention. For example, certain proteins may confer enhanced biological activity when present in a multimeric state (i.e., such enhanced activity may be due to the increased effective concentration of such proteins whereby more protein is available in a localized location).

ANTIBODY-BASED GENE THERAPY

In a specific embodiment, nucleic acids comprising sequences encoding antibodies or functional derivatives thereof, are administered to treat, inhibit or prevent a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or

expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., Clinical Pharmacy 12:488-505 (1993); Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62:191-217 (1993); May, TIBTECH 11(5):155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a preferred aspect, the compound comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989). In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids in vitro, then transplanted into the patient. These two approaches are known, respectively, as in vivo or ex vivo gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered in vivo, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, e.g., by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Pat. No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem . . . 262:4429-4432 (1987)) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188, WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989)).

In a specific embodiment, viral vectors that contains nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller et al., Meth. Enzymol. 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., Biotherapy 6:291-302 (1994), which describes the use of a retroviral vector to deliver the mdrl gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene

therapy are: Clowes et al., J. Clin. Invest. 93:644-651 (1994); Kiem et al., Blood 83:1467-1473 (1994); Salmons and Gunzberg, Human Gene Therapy 4:129-141 (1993); and Grossman and Wilson, Curr. Opin. in Genetics and Devel. 3:110-114 (1993).

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout et al., Human Gene Therapy 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., Science 252:431-434 (1991); Rosenfeld et al., Cell 68:143-155 (1992); Mastrangeli et al., J. Clin. Invest. 91:225-234 (1993); PCT Publication WO94/12649; and Wang, et al., Gene Therapy 2:775-783 (1995). In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Pat. No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated

gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, e.g., Loeffler and Behr, Meth. Enzymol. 217:599-618 (1993); Cohen et al., Meth. Enzymol. 217:618-644 (1993); Cline, Pharmac. Ther. 29:69-92m (1985) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (e.g., hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as Tlymphocytes, Blymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient. In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained in vitro can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598; Stemple and Anderson, Cell 71:973-985 (1992); Rheinwald, Meth. Cell Bio. 21A:229 (1980); and Pittelkow and Scott, Mayo Clinic Proc. 61:771 (1986)).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

THERAPEUTIC USES OF siRNA

The present invention is further directed to RNA interference (RNAi)-based therapies which involve administering short interference RNAs (siRNAs) or siRNA-expressing DNA construct of the invention to an animal, preferably a mammal, and most preferably a human, patient for treating one or more of the disclosed diseases, disorders, or conditions (Nature 2001, 411:494; Target 2003, 2:42;FEBS 2002, 527:274). Therapeutic compounds of the invention include, but are not limited to, siRNAs of the invention (including fragments, analogs and derivatives thereof as described herein) and nucleic acids homologous to siRNAs of the invention (including fragments, analogs and derivatives thereof and anti-idiotypic antibodies as described herein). The siRNAs of the invention can be used to treat, inhibit or prevent diseases, disorders or conditions associated with aberrant expression and/or activity of a polypeptide of the invention, including, but not limited to, any one or more of the diseases, disorders, or conditions described herein. The treatment and/or prevention of diseases, disorders, or conditions associated with aberrant expression and/or activity of a polypeptide of the invention includes, but is not limited to, alleviating symptoms associated with those diseases, disorders or conditions. Antibodies of the invention may be provided in pharmaceutically acceptable compositions as known in the art or as described herein.

A summary of the ways in which the siRNAs of the present invention may be used therapeutically includes binding polynucleotides of the present invention locally or systemically in the body or by direct cytotoxicity of the siRNA, e.g. as mediated by complement (CDC) or by effector cells (ADCC). Some of these approaches are described in more detail below. Armed with the teachings provided herein, one of ordinary skill in the art will know how to use the siRNAs of the present invention for diagnostic, monitoring or therapeutic purposes without undue experimentation.

The siRNAs of this invention may be advantageously utilized in combination with other monoclonal or chimeric antibodies, or with lymphokines or hematopoietic growth factors (such as, e.g., IL-2, IL-3 and IL-7), for example, which serve to increase the number or activity of effector cells which interact with the antibodies.

The siRNAs of the invention may be administered alone or in combination with other types of treatments (e.g., radiation therapy, chemotherapy, hormonal therapy, immunotherapy and anti-tumor agents). Generally, administration of products of a species origin or species reactivity (in the case of siRNAs) that is the same species as that of the patient is preferred. Thus, in a preferred embodiment, human antibodies, fragments derivatives, analogs, or nucleic acids, are administered to a human patient for therapy or prophylaxis.

The siRNAs directed against polypeptides of the present invention are useful for inhibiting allergic reactions in animals. For example, by administering a therapeutically acceptable dose of an siRNA, or siRNAs, of the present invention, or a cocktail of the present siRNAs, or in combination with other siRNAs of varying sources, the animal may not elicit an allergic response to antigens.

Likewise, one could envision cloning the gene encoding an siRNA directed against a polypeptide of the present invention, said polypeptide having the potential to elicit an allergic and/or immune response in an organism, and transforming the organism with said siRNA gene such that it is expressed (e.g., constitutively, inducibly, etc.) in the organism. Thus, the organism would effectively become resistant to an allergic response resulting from the ingestion or presence of such an immune/allergic reactive polypeptide. Moreover, such a use of the siRNAs of the present invention may have particular utility in preventing and/or ameliorating autoimmune diseases and/or disorders, as such conditions are typically a result of siRNAs being directed against endogenous proteins. For example, in the instance where the polypeptide of the present invention is responsible for modulating the immune response to auto-antigens, transforming the organism and/or individual with a construct comprising any of the promoters disclosed herein or otherwise known in the art, in addition, to a polynucleotide encoding the siRNA directed against the polypeptide of the present invention could effective inhibit the organisms immune system from eliciting an immune

response to the auto-antigen(s). Detailed descriptions of therapeutic and/or gene therapy applications of the present invention are provided elsewhere herein.

siRNA-BASED GENE THERAPY

In a specific embodiment, nucleic acids comprising sequences encoding siRNAs or functional derivatives thereof, are administered to treat, inhibit or prevent a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention, by way of gene therapy. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., Clinical Pharmacy 12:488-505 (1993); Wu and Wu, Biotherapy 3:87-95 (1991); Tolstoshev, Ann. Rev. Pharmacol. Toxicol. 32:573-596 (1993); Mulligan, Science 260:926-932 (1993); and Morgan and Anderson, Ann. Rev. Biochem. 62:191-217 (1993); May, TIBTECH 11(5):155-215 (1993). Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a preferred aspect, the compound comprises nucleic acid sequences encoding a siRNA, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989). In specific embodiments, the expressed antibody molecule is

a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids in vitro, then transplanted into the patient. These two approaches are known, respectively, as in vivo or ex vivo gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered in vivo, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing them as part of an appropriate nucleic acid expression vector and administering it so that they become intracellular, e.g., by infection using defective or attenuated retrovirals or other viral vectors (see U.S. Pat. No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering them in linkage to a peptide which is known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem . . . 262:4429-4432 (1987)) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188, WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination (Koller and Smithies, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989); Zijlstra et al., Nature 342:435-438 (1989)).

In a specific embodiment, viral vectors that contains nucleic acid sequences encoding a siRNA of the invention are used. For example, a retroviral vector can be used (see Miller et al., Meth. Enzymol. 217:581-599 (1993)). These

retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, which facilitates delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen et al., Biotherapy 6:291-302 (1994), which describes the use of a retroviral vector to deliver the mdrl gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes et al., J. Clin. Invest. 93:644-651 (1994); Kiem et al., Blood 83:1467-1473 (1994); Salmons and Gunzberg, Human Gene Therapy 4:129-141 (1993); and Grossman and Wilson, Curr. Opin. in Genetics and Devel. 3:110-114 (1993).

Adenoviruses are other viral vectors that can be used in gene therapy. Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout et al., Human Gene Therapy 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld et al., Science 252:431-434 (1991); Rosenfeld et al., Cell 68:143-155 (1992); Mastrangeli et al., J. Clin. Invest. 91:225-234 (1993); PCT Publication WO94/12649; and Wang, et al., Gene Therapy 2:775-783 (1995). In a preferred embodiment, adenovirus vectors are used.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy (Walsh et al., Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Pat. No. 5,436,146).

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes

the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells (see, e.g., Loeffler and Behr, Meth. Enzymol. 217:599-618 (1993); Cohen et al., Meth. Enzymol. 217:618-644 (1993); Cline, Pharmac. Ther. 29:69-92m (1985) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (e.g., hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes; blood cells such as Tlymphocytes, Blymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient. In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding a siRNA are introduced into the cells

such that they are expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained in vitro can potentially be used in accordance with this embodiment of the present invention (see e.g. PCT Publication WO 94/08598; Stemple and Anderson, Cell 71:973-985 (1992); Rheinwald, Meth. Cell Bio. 21A:229 (1980); and Pittelkow and Scott, Mayo Clinic Proc. 61:771 (1986)).

DIAGNOSIS AND IMAGING WITH ANTIBODIES

Labeled antibodies, and derivatives and analogs thereof, which specifically bind to a polypeptide of interest can be used for diagnostic purposes to detect, diagnose, or monitor diseases, disorders, and/or conditions associated with the aberrant expression and/or activity of a polypeptide of the invention. The invention provides for the detection of aberrant expression of a polypeptide of interest, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of aberrant expression.

The invention provides a diagnostic assay for diagnosing a disorder, comprising (a) assaying the expression of the polypeptide of interest in cells or body fluid of an individual using one or more antibodies specific to the polypeptide interest and (b) comparing the level of gene expression with a standard gene expression level, whereby an increase or decrease in the assayed polypeptide gene expression level compared to the standard expression level is indicative of a particular disorder. With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

Antibodies of the invention can be used to assay protein levels in a biological sample using classical immunohistological methods known to those of skill in the art (e.g., see Jalkanen, et al., J. Cell. Biol. 101:976-985 (1985); Jalkanen, et al., J. Cell. Biol. 105:3087-3096 (1987)). Other antibody-based methods useful for detecting protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). Suitable antibody assay labels are known in the art and include enzyme labels, such as, glucose oxidase; radioisotopes, such as iodine (125I, 121I), carbon (14C), sulfur (35S), tritium (3H), indium (112In), and technetium (99Tc); luminescent labels, such as luminol; and fluorescent labels, such as fluorescein and rhodamine, and biotin.

One aspect of the invention is the detection and diagnosis of a disease or disorder associated with aberrant expression of a polypeptide of interest in an animal, preferably a mammal and most preferably a human. In one embodiment, diagnosis comprises: a) administering (for example, parenterally, subcutaneously, or intraperitoneally) to a subject an effective amount of a labeled molecule which specifically binds to the polypeptide of interest; b) waiting for a time interval following the administering for permitting the labeled molecule to preferentially concentrate at sites in the subject where the polypeptide is expressed (and for unbound labeled molecule to be cleared to background level); c) determining background level; and d) detecting the labeled molecule in the subject, such that detection of labeled molecule above the background level indicates that the subject has a particular disease or disorder associated with aberrant expression of the polypeptide of interest. Background level can be determined by various methods including, comparing the amount of labeled molecule detected to a standard value previously determined for a particular system.

It will be understood in the art that the size of the subject and the imaging system used will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of 99 mTc. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain the specific protein. In vivo tumor imaging is described in S. W. Burchiel et al., "Immunopharmacokinetics of

Radiolabeled Antibodies and Their Fragments." (Chapter 13 in Tumor Imaging: The Radiochemical Detection of Cancer, S. W. Burchiel and B. A. Rhodes, eds., Masson Publishing Inc. (1982).

Depending on several variables, including the type of label used and the mode of administration, the time interval following the administration for permitting the labeled molecule to preferentially concentrate at sites in the subject and for unbound labeled molecule to be cleared to background level is 6 to 48 hours or 6 to 24 hours or 6 to 12 hours. In another embodiment the time interval following administration is 5 to 20 days or 5 to 10 days.

In an embodiment, monitoring of the disease or disorder is carried out by repeating the method for diagnosing the disease or disease, for example, one month after initial diagnosis, six months after initial diagnosis, one year after initial diagnosis, etc.

Presence of the labeled molecule can be detected in the patient using methods known in the art for in vivo scanning. These methods depend upon the type of label used. Skilled artisans will be able to determine the appropriate method for detecting a particular label. Methods and devices that may be used in the diagnostic methods of the invention include, but are not limited to, computed tomography (CT), whole body scan such as position emission tomography (PET), magnetic resonance imaging (MRI), and sonography.

In a specific embodiment, the molecule is labeled with a radioisotope and is detected in the patient using a radiation responsive surgical instrument (Thurston et al., U.S. Pat. No. 5,441,050). In another embodiment, the molecule is labeled with a fluorescent compound and is detected in the patient using a fluorescence responsive scanning instrument. In another embodiment, the molecule is labeled with a positron emitting metal and is detected in the patent using positron emission-tomography. In yet another embodiment, the molecule is labeled with a paramagnetic label and is detected in a patient using magnetic resonance imaging (MRI).

RECEPTOR EXPRESSION MODULATION

In yet another aspect, the present invention provides a method for blocking or modulating the expression of a cellular GPR91 receptor by interfering with the transcription or translation of a DNA or RNA polynucleotide encoding the GPR91

activating receptor. The method comprises exposing a cell capable of expressing a GPR91 receptor to a molecule that interferes with the proper transcription or translation of a DNA or RNA polynucleotide encoding the GPR91 activating receptor. The molecule can be an organic molecule, a bioorganic molecule, an antisense nucleotide, an RNAi nucleotide, or a ribozyme.

In a preferred embodiment, the method comprises blocking or modulating the expression of cellular GPR91 receptor by exposing a cell to a polynucleotide that is antisense to or forms a triple helix with GPR91 activating receptor-encoding DNA or with DNA regulating expression of GPR91 activating receptor-encoding DNA. The cell is exposed to antisense polynucleotide or triple helix-forming polynucleotide in an amount sufficient to inhibit or regulate expression of the GPR91 activating receptor. Also, the present invention provides a method for blocking or modulating expression of GPR91 receptor in an animal by administering to the animal a polynucleotide that is antisense to or forms a triple helix with GPR91 activating receptor-encoding DNA or with DNA regulating expression of GPR91 activating receptor-encoding DNA. The animal is administered antisense polynucleotide or triple helix-forming polynucleotide in an amount sufficient to inhibit or regulate expression of GPR91 receptor in the animal. Preferably, the antisense polynucleotide or triple helix-forming polynucleotide is a DNA or RNA polynucleotide.

Methods for exposing cells to antisense polynucleotides and for administering antisense polynucleotides to animals are well known in the art. In a preferred method, the polynucleotide is incorporated into the cellular genome using know methods and allowed to be expressed inside the cell. The expressed antisense polynucleotide binds to polynucleotides coding for GPR91 receptor and interferes with their transcription or translation.

The methods are useful for inhibiting cytokine and receptor expression while conducting research on various types of cells, e.g., neutrophils or mast cells, and for preventing or treating animal disease characterized by excess cytokine production compared to non-disease states.

DISEASE PREDISPOSITION DIAGNOSTIC

In another aspect, the present invention provides a method for diagnosing the predisposition of a patient to develop diseases caused by the unregulated

expression of cytokines. The invention is based upon the discovery that the presence of or increased amount of GPR91 receptor in certain patient cells, tissues, or body fluids indicates that the patient is predisposed to certain immune diseases. In one embodiment, the method comprises collecting a cell, tissue, or body fluid sample known to contain few if any GPR91 receptor from a patient, analyzing the tissue or body fluid for the presence of GPR91 receptor in the tissue, and predicting the predisposition of the patient to certain immune diseases based upon the level of expression of GPR91 receptor in the tissue or body fluid. In another embodiment, the method comprises collecting a cell, tissue, or body fluid sample known to contain a defined level of GPR91 receptor from a patient, analyzing the tissue or body fluid for the amount of GPR91 receptor in the tissue, and predicting the predisposition of the patient to certain immune diseases based upon the change in the amount of GPR91 receptor in the tissue or body fluid compared to a defined or tested level established for normal cell, tissue, or bodily fluid. The defined level of GPR91 receptor may be a known amount based upon literature values or may be determined in advance by measuring the amount in normal cell, tissue, or body fluids. Specifically, determination of GPR91 receptor levels in certain tissues or body fluids permits specific and early, preferably before disease occurs, detection of immune diseases in the patient. Immune diseases that can be diagnosed using the present method include, but are not limited to, the immune diseases described herein. In the preferred embodiment, the tissue or body fluid is peripheral blood, peripheral blood leukocytes, biopsy tissues such as lung or skin biopsies, and synovial fluid and tissue.

DISEASE PREVENTION AND TREATMENT

In another aspect, the present invention provides a method for preventing or treating GPR91 protein mediated diseases in a mammal. The method comprises administering a disease preventing or treating amount of a GPR91 receptor agonist or antagonist to the mammal. The agonist or antagonist binds to the GPR91 receptor and regulates cytokine and cellular receptor expression to produce cytokine levels characteristic of non-disease states. Preferably, the disease is an allergy, asthma, autoimmune, or other inflammatory disease. Most preferably, the disease is an allergy or asthma.

The dosages of GPR91 receptor agonist or antagonist vary according to the age, size, and character of the particular mammal and the disease. Skilled artisans can determine the dosages based upon these factors. The agonist or antagonist can be administered in treatment regimes consistent with the disease, e.g., a single or a few doses over one to several days to ameliorate a disease state or periodic doses over an extended time to prevent allergy or asthma.

The agonists and antagonists can be administered to the mammal in any acceptable manner including oral administration, by injection, using an implant, aerosol into the lungs and the like. Injections and implants permit precise control of the timing and dosage levels used for administration. The agonists and antagonists may be administered parenterally. As used herein parenteral administration means by intravenous, intramuscularly, or intraperitoneal injection, or by subcutaneous implant.

When administered by injection, the agonists and antagonists can be administered to the mammal in an injectable formulation containing any biocompatible agent and agonists and antagonists compatible carrier such as various vehicles, adjuvants, additives, and diluents. Aqueous vehicles such as water having no nonvolatile pyrogens, sterile water, and bacteriostatic water are also suitable to form injectable solutions. In addition to these forms of water, several other aqueous vehicles can be used. These include isotonic injection compositions that can be sterilized such as sodium chloride, Ringer's, dextrose, dextrose and sodium chloride, and lactated Ringer's. Nonaqueous vehicles such as cottonseed oil, sesame oil, or peanut oil and esters such as isopropyl myristate may also be used as solvent systems for the compositions. Additionally, various additives which enhance the stability, sterility, and isotonicity of the composition including antimicrobial preservatives, antioxidants, chelating agents, and buffers can be added. Any vehicle, diluent, or additive used would, however, have to be biocompatible and compatible with the agonists and antagonists according to the present invention.

THERAPEUTIC OR PROPHYLACTIC ACTIVITY

The compounds or pharmaceutical compositions of the invention are preferably tested in vitro, and then in vivo for the desired therapeutic or prophylactic activity, prior to use in humans. For example, in vitro assays to

demonstrate the therapeutic or prophylactic utility of a compound or pharmaceutical composition include, the effect of a compound on a cell line or a patient tissue sample. The effect of the compound or composition on the cell line and/or tissue sample can be determined utilizing techniques known to those of skill in the art including, but not limited to, rosette formation assays and cell lysis assays. In accordance with the invention, in vitro assays which can be used to determine whether administration of a specific compound is indicated, include in vitro cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a compound, and the effect of such compound upon the tissue sample is observed.

THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment, inhibition and prophylaxis by administration to a subject of an effective amount of a compound or pharmaceutical composition of the invention, preferably an antibody or an siRNA of the invention. In a preferred aspect, the compound is substantially purified (e.g., substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human.

Formulations and methods of administration that can be employed when the compound comprises a nucleic acid or an immunoglobulin are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a compound of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, e.g., Wu and Wu, J. Biol. Chem.. 262:4429-4432 (1987)), construction of a nucleic acid as part of a retroviral or other vector, etc.

Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds or compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal

mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compounds or compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compounds or compositions of the invention locally to the area in need of treatment; this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. Preferably, when administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid.).

In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J., Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J.

Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment where the compound of the invention is a nucleic acid encoding a protein, the nucleic acid can be administered in vivo to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Pat. No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., Proc. Natl. Acad. Sci. USA 88:1864-1868 (1991)), etc. Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a compound, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol,

water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E. W. Martin. Such compositions will contain a therapeutically effective amount of the compound, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with anions such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with cations such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the compound of the invention which will be effective in the treatment, inhibition and prevention of a disease or disorder associated with aberrant expression and/or activity of a polypeptide of the invention can be determined by standard clinical techniques. In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

For antibodies, the dosage administered to a patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign polypeptides. Thus, lower dosages of human antibodies and less frequent administration is often possible. Further, the dosage and frequency of administration of antibodies of the invention may be reduced by enhancing uptake and tissue penetration (e.g., into the brain) of the antibodies by modifications such as, for example, lipidation.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

GPR91 POLYPEPTIDE PURIFICATION

The antibodies of the present invention may also be used in a method for isolating and purifying GPR91 receptor from recombinant cell cultures, contaminants, and native environments. The method comprises exposing a composition containing GPR91 receptor and contaminants to an antibody capable of binding to the receptors, allowing the GPR91 receptor to bind to the antibody,

separating the antibody-receptor complexes from the contaminants, and recovering the GPR91 receptor from the complexes. Various purification methods known in the art may be used, e.g., affinity purification methods that recover GPR91 receptor from recombinant cell culture or native sources. In this method, the antibodies against GPR91 receptor are immobilized on a suitable support such a Sephadex resin or filter paper using methods well known in the art. The immobilized antibody then is contacted with a sample composition containing the GPR91 receptor to be purified and contaminants. The support is then washed with a suitable solvent capable of removing substantially all the material in the sample except the GPR91 receptor bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that that removes the GPR91 receptor from the antibody.

KNOCKOUT ANIMALS

In another aspect, the present invention provides a knockout animal comprising a genome having a heterozygous or homozygous disruption in its endogenous GPR91 receptor gene that suppresses or prevents the expression of biologically functional GPR91 receptor proteins. Preferably, the knockout animal of the present invention has a homozygous disruption in its endogenous GPR91 receptor gene. Preferably, the knockout animal of the present invention is a mouse. The knockout animal can be made easily using techniques known to skilled artisans. Gene disruption can be accomplished in several ways including introduction of a stop codon into any part of the polypeptide coding sequence that results in a biologically inactive polypeptide, introduction of a mutation into a promoter or other regulatory sequence that suppresses or prevents polypeptide expression, insertion of an exogenous sequence into the gene that inactivates the gene, and deletion of sequences from the gene.

Several techniques are available to introduce specific DNA sequences into the mammalian germ line and to achieve stable transmission of these sequences (transgenes) to each subsequent generation. The most commonly used technique is direct microinjection of DNA into the pronucleus of fertilized oocytes. Mice or other animals derived from these oocytes will be, at a frequency of about 10 to 20%, the transgenic founders that through breeding will give rise to the different transgenic mouse lines. Methods for generating transgenic animals via embryo

manipulation and microinjection, particularly animals such as mice, have become conventional in the art, e.g., U.S. Pat. Nos. 4,736,866, 4,870,009, and 4,873,191 and in Hogan, B., Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals.

Embryonic stem cell ("ES cell") technology can be used to create knockout mice (and other animals) with specifically deleted genes. Totipotent embryonic stem cells, which can be cultured in vitro and genetically modified, are aggregated with or microinjected into mouse embryos to produce a chimeric mouse that can transmit this genetic modification to its offspring. Through directed breeding, a mouse can thus be obtained that lacks this gene. Several other methods are available for the production of genetically modified animals, e.g., the intracytoplasmic sperm injection technique (ICSI) can be used for transgenic mouse production. This method requires microinjecting the head of a spermatocyte into the cytoplasm of an unfertilized oocyte, provoking fertilization of the oocyte, and subsequent activation of the appropriate cellular divisions of a preimplantation embryo. The mouse embryos thus obtained are transferred to a pseudopregnant receptor female. The female will give birth to a litter of mice. In ICSI applied to transgenic mouse production, a sperm or spermatocyte heads suspension is incubated with a solution containing the desired DNA molecules (transgene). These interact with the sperm that, once microinjected, act as a carrier vehicle for the foreign DNA. Once inside the oocyte, the DNA is integrated into the genome, giving rise to a transgenic mouse. This method renders higher yields (above 80%) of transgenic mice than those obtained to date using traditional pronuclear microinjection protocols.

EXPERIMENTAL METHODS

I. Cell Culture

Human cord blood $CD34^+$ cells (Bio-Whittaker, Walkersville, MD) were cultured up to 9 weeks in culture media consisting of RPMI1640 (Invitrogen) supplemented with 20% FBS (Sigma-Aldrich, St. Louis, MO), 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml gentamicin, 80 ng/ml SCF, 50 ng/ml IL-6 and 5 ng/ml IL-10. Cells were stained with anti-tryptase mAb to determine the percentage of mast cells. Cell suspensions were seeded at

a density of 5 X 10^5 cells/ml and cytokine-supplemented medium was replaced once a week. Recombinant human IgE was used for IgE cross-linking experiment. Other cell lines were cultured following ATCC's recommendations.

II. Real Time Quantitative PCR

Real-time quantitative PCR was performed with the ABI Prism 7900 (Applied Biosystems, Inc.) sequence detection system, using CYBR Green reagents, according to the manufacture's instructions. Total RNAs were isolated to measure the level of GPR91 mRNA in the following cells: Daudi (a B lymphoblast cell line derived from Burkitt's lymphoma, ATCC No. CCL-213), THP-1 (a monocytic leukemia cell line, ATCC No. TIB202), HMC-1, (a mastoma cell line); peripheral blood mononuclear cells (PBMC); primary monocytes; primary B cells; primary neutrophils; *in vitro* cultured mast cells at week 8-9. The first strand cDNA from brain, heart, kidney, liver, lung, spleen, thymus and trachea were from DB Bioscience Clontech (Palo Alto, CA).

Equal amounts of each of the RNAs from the cells indicated above were used in reverse transcriptase reaction to generate first strand cDNAs, which were used as templates in quantitative PCR reactions to obtain the threshold amplification cycle (C_t). The C_t was normalized using the control C_t from 18S RNAs to obtain ΔC_t. To compare relative levels of gene expression of GPR91 in different cells and tissues, $\Delta\Delta C_t$ values were calculated by using the lowest expression level as the base, which were then converted to the values of relative expression difference.

III. Transient Transfection and Luciferase Reporter Assay

Transient transfection was performed using Lipofectamine 2000 system (Invitrogen, Carlsbad, CA). For Western blot analysis, twenty micrograms of plasmid DNA was transfected into 293T cells in a 100 mm tissue culture dish; and 40 hours later, the cells were harvested in PBS-based, enzyme-free cell dissociation buffer (Invitrogen) and processed as described in the following sections. For luciferase reporter assay, GPR91 expression construct was cotransfected in HMC-1 cells with Mercury Pathway Profiling reporter plasmids, or with luciferase reporter plasmids for monitering mast cell activation (DB BioScience Clontech, Polo Alto, CA) and a control luciferase plasmid, pRL-SChA. The cells were harvested 40 hours later, and assayed for luciferase activity using

the dual luciferase assay kit following manufacturer's protocol (Promega, Madison, WI).

The firefly luciferase reporter constructs for monitering mast cell activation were generated by inserting the PCR-amplified promoter sequences from human IL8, IL13, TNF-α, tryptase β1, tryptase β2, FcεRIα and genes into the promoterless luciferase reporter plasmid vector, TA-Luc (DB Bioscience Clontech). Those promoters were chosen because of their pivotal role in immune responses.

IV. Protein Extraction and Western Blot Analysis

The whole cell protein sample was prepared by resuspending cells in ddH_2O, and heated at 98°C for 5 minutes after adding equal volume of 2 X sample loading buffer. To separate membrane fraction from soluble fraction, cells were subject to lysis procedure. Briefly, cells were suspended in the buffer containing 20 mM Tris-HCl (pH7.6), 70 mM KCl and 1 mM EDTA and lysed by three freeze-thaw cycles. Insoluble membrane fraction was separated from soluble proteins by centrifugation at maximum speed in a microcentrifuge. The proteins were fractionated in a 4-15% gradient SDS-PAGE. Western blot was performed as previously described (Li, K. 2000 Cell 101:389-399) by using anti-Flag (Sigma) or anti-V5 mAb (Invitrogen).

EXAMPLES

This invention can be further illustrated by the following examples, although it will be understood that these examples are included merely for purposes of illustration and are not intended to limit the scope of the invention unless otherwise specifically indicated.

EXAMPLE 1

IDENTIFICATION OF MAST CELL-DIFFERENTIALLY EXPRESSED GPRC

Microarray technology was used to compare gene expression quantitatively between Mast Cells, THP1 and PBMC, and identify those genes preferentially expressed (>2.5 fold) in Mast Cells versus THP1 and PBMC.

Mast cells were cultured according to the experimental method described in section (I) above. Cells were depleted for CD14 and CD15 expressing cells by the use of immunological magnetic beads (Dynal) and the remaining cells were harvested at week 8. PBMC cells were isolated from healthy donor blood by

gradient centrifugation based on Amersham's Ficoll-Paque lymphocyte isolation procedure. Human THP1 cells were cultured in media RPMI1640 with 10% FBS. RNA was isolated from each of these cell types by using a Qiagen Total RNA isolation kit according to the manufaturer's instructions.

GeneChip expression analysis was done using the Affymetrix expression assay service. Affymetric's standard array (GeneChip Human Genome U133 Set) is composed of two microarrays containing over 1 million unique oligonucleotides covering more than 39,000 transcript variants which in turn represent greater than 33,000 human genes.

High quality total RNA from each of Mast cells, THP1, and PBMC cultures was used to obtain biotin-labeled cRNA. Single-stranded cDNA was synthesized by reverse transcription using poly(A) RNA present in the total RNA sample and then converted to double-strnaded cDNA. An *in vitro* transcription reaction was performed in the presence of biotinylated UTP and CTP to produce biotin-labeled cRNA. cRNA was then fragmented in the presence of heat and Mg^{2+}. The fragmented cRNA from each cell type was hybridized to a test array to assess target quality and labeling efficiency. The test array was then washed, stained with streptavidin-phycoerythrin, and scanned using a GeneArray scanner. Images were analyzed using quality control parameters such as 3'/5' ratio of housekeeping genes, presence of spiked control cRNA sequences, noise (Q values), scaling factors, etc. After assessment, the cRNA was hybridized to the U133 standard array for 16 hours at 45 °C. The array was then washed, stained with streptavidin-phycoerythrin, and scanned using a GeneArray scanner. The expression data obtained was extracted from black and white images using Affymetrix MAS 5.0 software, and statistical algorithms were employed to calculate a quantitative value (Signal Intensity) and a qualitative value (presence or absence) for each transcript on the array.

Comparative analysis among the mast cell, PBMC and THP-1 cells of the human GeneChip microarray data showed that a G protein-coupled receptor (GPCR) of unknown function is highly expressed in the *in vitro*-cultured, primary mast cells, but only expressed at low levels in PBMC and HTP-1 cells.

A database comparison revealed that this GPCR (GenBank accession no. NM_348078, BC030948, AF348078, AC068647 and other homologous sequences) was termed GPR91.

The BLAST sequence similarity search against the public GenBank database revealed that GPR91 shares significant homology to P2Y purinergic receptors and cysteinyl leukotriene receptors. For examples, the pair-wised comparison using the computer algorism, DNAStar, showed that GPR91 shares 29.9% to 35.7% of amino acid sequence identity to the six known members of P2Y receptor family, and that it also shares 29.3% to 31.6% of the sequence identity to the cysteinyl leukotriene receptors, CysLT1 and CysLT2. Prediction of the coupling specificity of GPR91 to its G protein indicated that it is most likely involved in interacting with Gαq/11 subtype. This prediction was based on the fact that the specificity of the receptor-G protein interaction is governed by the intracellular domains of the receptor. A data-mining approach combining pattern discovery with membrane topology prediction was used to find patterns of amino acid residues in the intracellular domains of GPCR sequences that are specific for coupling to a particular functional class of G proteins.

EXAMPLE 2

REAL-TIME QUANTITATIVE PCR ANALYSIS OF GPR91 MRNA EXPRESSION

Two oligonucleotide primers: 5'TACTGCTCTGCCCCTTGAAAA-3' (SEQ ID NO 4) and 5'ACCACTGCCATGATGACCAA-3' (SEQ ID NO 5),

were synthesized from the GPR91 nucleotide sequences following selection using Primer Express 2.0 (Applied Biosystems, Inc.), and then used to monitor the expression of GPR91. The reactions were performed according to the method(s) described in section II above. The quantitative RT-PCR analysis showed that GPR91 mRNA was expressed at highest level in human mast cells, and was expressed at moderate levels in kidney, spleen, THP-1, and PBMC (Table 1).

TABLE 1

Expression profile of GPR91 mRNA assessed by quantitative RT-PCR

Tissue/Cell	Ct	Relative xpression
Brain	30.5	119.2

Heart	29.1	318.9
Kidney	24.3	8321.1
Liver	29.9	167.1
Lung	31.4	63.2
Spleen	27.1	1144.8
Thymus	30.3	130.4
Traachea	29.4	250.1
HPB-All	33.4	24.7
Monocyte	33.9	14.0
Monocyte (6/20/02)	33.0	38.4
PBMC (9/28/01)	29.2	409.4
PBMC (5/9/02)	29.6	306.1
Neutrophil (5/9/02)	30.5	170.1
Neutrophil (6/20/02)	31.1	138.3
Mast Cell 1898 (6/10/02)	22.9	32690.5
Mast Cell 2128 (5/9/02)	23.1	27220.5
THP- 1	25.9	3835.2
Daudi	31.3	91.2
HMC-1	37.9	1.0

EXAMPLE 3

EXPRESSION CONSTRUCTS OF GPR91

The coding sequence of GPR91 (SEQ ID NO 1) was amplified by PCR from mast cell RNA and cloned into pcDNA3.1TOPO (Invitrogen, Carlsbad, CA). The sequence of GPR91 expression construct was verified to be identical to NM_348078, BC030948, AF348078, and AC068647 (GenBank Accession Number). A number of mutant GPR91 expression constructs were constructed by inserting epitope tag at various position of the coding region. One of the insertion mutations, GPR91-20Flag (SEQ ID NO 3), showed much stronger activity than the wild type GPR91 in GPR91-luciferase reporter assay (Table 2). This constitutively active mutant was proven useful to unravel the downstream signal transduction and gene activation. The construct, GPR91-20Flag, was generated by PCR SOEing (Ho et al., 1989 Gene 77:51-59; Horton et al., 1990 Biotechniques 8:528-

535), which contains a Flag tag sequence inserted after the first 20 amino acid residues (SEQ ID NO 3 and SEQUENCE 2).

TABLE 2

Luciferase assay of the wild type and tagged GPR91 expression constructs co-transfected with Luc reporter plasmids

Luciferase reporters	pcDNA3.1		GPR91		GPR91-20Flag	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
TA-Luc	0.414	0.307	0.903	0.421	1.667	0.096
NFAT-Luc	3.501	2.621	90.303	20.012	435.213	23.351

EXAMPLE 4

ACTIVATION OF NFAT AND AP1 SIGNALING PATHWAYS BY GPR91

The intracellular signaling pathways activated by GPR91 were investigated using the luciferase reporter assay described in Section III above. Among the Mercury Pathway Profiling luciferase reporters (DB BioScience Clontech) used in our assays, the luciferase reporters, NFAT-Luc and AP1-Luc, were activated ~20 fold and ~5 fold, respectively, by GPR91wt in two independent experiments (Table 3). Much stronger activation of NFAT-Luc reporter was observed with the insertion mutation construct, GPR91-20Flag, which showed more than 3 fold higher activity than GPR91 (Table 2). These results indicated that the expression of GPR91 in the stable line of human mast cell, HMC-1, activated the intracellular signaling pathways mediated through the transcription factors, NFAT and AP1. To confirm that GPR91 activates the signaling pathway mediated through the intracellular calcium flux to calcineurin and GPR91 activation, we tested whether the well-known calcineurin inhibitor, cyclosporin A could block the NFAT activation by GPR91. As shown in Table 4, the stimulatory effect of GPR91 on NFAT-Luc reporter was reduced by 82-87% at micro molar or submicro molar concentrations of cyclosporin A. These evidences confirm that GPR91 activates the transcription factor, NFAT, through the intracellular calcium flux and calcineurin activation. The process may start with GPR91 recruiting the Gαq/11 (or q/11-like) subunit, which in turn activates the G protein and phospholipase C β and results in intracellular calcium flux.

TABLE 3

Luciferase assay of GPR91 expression construct co-transfected with Luc reporter plasmids

		Luciferase reporter plasmid						
		TA-Luc	NFkB	NFAT	GAS	STAT3	AP1	Myc
pcDNA3.1	Mean	0.59	2.89	4.98	25.59	16.32	3.23	10.23
	S.D.	0.39	0.25	3.42	7.71	4.98	0.41	6.35
GPR91wt	Mean	1.21	5.28	100.5	30.37	14.27	16.99	9.09
	S.D.	0.36	0.45	6.65	5.7	0.27	1.31	3.89

TABLE 4

Inhibition of GPR91 activity by cyclosporin A

	GPR91 + NFAT-Luc,				pcDNA3.1 + NFAT-Luc
Cyclosporin A	0 uM	0.5 uM	1 uM	2 uM	
Mean	99.98	20.29	23.85	22.08	8.09
S.D.	15.45	0.90	4.35	6.33	1.07

EXAMPLE 5

GPR91 ENHANCES THE CYTOKINE AND TRYPTASE PROMOTER ACTIVITY

The cytokine secretion and tryptase release are the hallmarks of mast cell activation, which are accompanied with increased *de novo* synthesis of the released proteins. To investigate the effects of GPR91 on the *de novo* synthesis and promoter activation of tryptases and cytokines, we performed transient transfection luciferase assay in HMC-1 cells using the firefly luciferase reporters containing tryptase β1, tryptase β2, IL8. IL13, TNFα, FcεRIα and promoters. The results (Table 5) showed that when co-transfected with GPR91-20Flag, the IL8 and IL13 luciferase reporter activity was increased up to 6.5 to 7.2 fold in comparison to the co-transfection with vector, pcDNA3.1, and that TNFα, tryptase β1, and β2 luciferase reporter activities increased up to 2.5 to 3.5 fold (Table 5).

TABLE 5

Luciferase reporter assay for the activation of tryptase and cytokine gene promoters by GPR91-20Flag

Luciferase reporters	pcDNA3.1		GPR91-20Flag	
	Mean	S.D.	Mean	S.D.
TA-Luc	0.805	0.021	1.662	0.163
FcεRIα	5.407	2.144	5.863	0.287
TRPβ1	32.144	8.839	83.755	18.584
TRPβ2	9.062	0.834	26.555	1.474
IL8	9.751	4.130	64.308	15.806
IL-13	3.603	26.043	26.043	8.409
TNFα	18.622	5.783	64.352	12.258

EXAMPLE 6

Methods for screening and identifying GPR91 ligand

There are a number of methods may be used to screen or to identify the ligand(s) for GPR91. These methods are either based on the measurement of intracellular calcium flux or GTP binding to G*α* subunits that are triggered by ligand binding to GPR91. These methods are briefly described as follows:

A. Calcium Imaging Assay For Ligand Binding To GPCR

Cells (such as CHO, 293T and NIH3T3) were transiently or stably transfected with the candidate GPCR, and seeded in high through put cell culture plate (96- or 384-wells). Then cells were loaded with calcium-sensitive, fluorescent dye indicator such as 4-(6-Aceoxymethoxy-2,7-dichloro-3-oxo-9-xanthenyl)-4'-methyl-2,2'-(ethylenedioxy) dianiline-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl)ester (Fluo 3-AM) or Fura-2 pentakis (acetoxymethyl)ester (Fura 2-AM). After adding compounds to the cell culture wells, the fluorescent signals could be measured by fluorometric imaging plate reader (FLIPR) or other

fluorescent signal-detecting device. (References: J. Biol. Chem. 2001, 276: 8608-8615; J. Biomol. Screening 2002, 7: 233-246).

B. M lanophore Assay For Ligand Binding To GPCR

Melanophores are Xenopus cells containing pigment granules (melanosomes), whose movement in the cytoplasm is affected by GPCR activity and second messenger levels of cAMP and diacylglycerol. When a GPCR is activated by its ligand, it in turn may activate Gq or Gs, which causes melanosomes undergoing rapid dispersion throughout the cell and the cell appearing dark. Xenopus melanophores may be transfected with GPR91, and subject to agonist compound treatment. The melanosome movement and cell color changes may be detected by a microplate reader or video image system. (References: J. Biol. Chem. 1993, 268: 5957-5964; J. Biol. Chem. 1999, 274: 8597-8603). This screening technique has been described in PCT WO92/01810 and is incorporated by reference.

C. Aequorin Assay For Ligand Binding To GPCR

Cells (such as CHO, 293T and NIH3T3) are transiently or stably co-transfected with the candidate GPCR and aequorin expression plasmids, and incubated with coelenterazine, a cofactor of aequorin, which interacts with apoaequorin to form aequorin. When the transfected cells stimulated with agonist compound(s), aequorin will emit light in response to increase of intracellular calcium level, which could be detected by a luminometer. (References: Cell Calcium 1993, 14: 663-671; Analytical Biochem. 1993, 209: 343-347;).

D. Guanine Nucleotide Exchange Assay For Ligand Binding To GPCR

The α subunit of G protein in non-active state bound with GDP is tightly associated with $\beta\gamma$ subunits that are targeted to plasma membrane. Ligand binding to GPCR triggers activation of the heterotrimeric G protein by virtue of guanine nucleotide exchange, converting the α subunit into a GTP-bound state which is recruited to the GPCR. The reaction was well documented being used to assess GPCR and ligand interaction. Briefly, the membrane fraction of the cells transfected with a specific GPCR was isolated and incubated with a labeled GTP such as 35S-GTPγS (guanosine 5'-3-O-(thio) triphosphate) or Eu-GTP (PerkinElmer Life Science). After washing, the GTP bound to the GPCR in the membrane fraction was determined by filter binding assay, and detected by

radioactivity scintillation counter or fluorescence plate reader. (References: J. Biol. Chem. 2002, 277: 31459-31465; J. Biol. Chem. 1990, 265: 18707-18712).

E. Transcription Factor (NFAT)-Bas d Reporter Assay For Ligand Binding To GPCR

Nuclear factor of activation in T cell (NFAT) may be activated by agonist binding to GPR91 and subsequent calcium flux (References: Cell 2002, 109: S67-S79; Ann. Rev. Immunol. 1997, 15: 707-747). This feature may be utilized to screen ligand or agonist compound if NFAT is linked to a reporter system, such as luciferase (DB BioSciences Clontech, Palo Alto, CA), β-lactamase or green fluorescent protein (DB BioSciences Clontech, Palo Alto, CA). (Reference: Yang J. et al. 2003, J. Biol. Chem. 278, in press).

F. Potassium-Dependent Current Assay For Ligand Binding To GPCR

Xenopus oocytes (or mammalian myocytes) may be micro-injected with RNAs that encode for GPCR and potassium channels (Kir3.1-3.4). If a ligand binds to the GPCR expressed on the oocyte surface, it will activate the GPCR, resulting in the release of G$\beta\gamma$ subunits from the heterotrimeric G protein and subsequent activation of the potassium channel. The current may be recorded by electrographic recorder. (References: Nature 2001, 409: 202-205; J. Biol. Chem. 2000, 275: 30531-30536; J. Biol. Chem. 1995, 270: 29059-29062).

G. TITLE

Another method involves co-transfecting HEK-293 cells with a mammalian expression plasmid encoding a GPR91, along with a mixture comprised of mammalian expression plasmids cDNAs encoding GU15 (Wilkie T. M. et al Proc Natl Acad Sci USA 1991 88: 10049-10053), GU16 (Amatruda T. T. et al Proc Natl Acad Sci USA 1991 8: 5587-5591, and three chimeric G-proteins refered to as Gqi5, Gqs5, and Gqo5 (Conklin B R et al Nature 1993 363: 274-276, Conklin B. R. et al Mol Pharmacol 1996 50: 885-890). Following a 24h incubation the transfected HEK-293 cells are plated into poly-D-lysine coated 96 well black/clear plates (Becton Dickinson, Bedford, Mass.).

The cells are assayed on FLIPR (Fluorescent Imaging Plate Reader, Molecular Devices, Sunnyvale, Calif.) for a calcium mobilization response following addition of test ligands. Upon identification of a ligand which stimulates calcium mobilization in HEK-293 cells expressing a given GPR and the G-protein

mixtures, subsequent experiments are performed to determine which, if any, G-protein is required for the functional response. HEK-293 cells are then transfected with GPR91, or co-transfected with GPR91 and G015, GD16, GqiS, Gqs5, or Gqo5. If GPR91 requires the presence of one of the G-proteins for functional expression in HEK-293 cells, all subsequent experiments are performed with HEK-293 cell cotransfected with the G-protein which gives the best response. Alternatively, the receptor can be expressed in a different cell line, for example RBL-2H3, without additional G proteins.

nouncement of approval by the FDA.

We Claim:

1. A method of screening for an compound that modulates of GPR91 receptor activity comprising:
    a. preparing a transfected cell comprising a nucleic acid sequence that encodes the amino acid sequence of SEQ ID NO 2;
    b. contacting transfected cell(s) with at least one compound whose ability to modulate the GPR91 receptor activity is sought to be determined;
    c. monitoring said cell for a compound that modulates the receptor's activity.
2. The method according to claim 1, wherein the cell is stably transfected.
3. The method according to claim 1, wherein the cell is transiently transfected.
4. The method according to claim 3, wherein the cell is a mast cell.
5. The method according to claim 1, wherein the compound is an agonist.
6. The method according to claim 1, wherein the compound is an antagonist.
7. The method according to claim 1, wherein the amount of calcium influx is monitored.
8. The method according to claim 1, wherein the cells employed in step (a) further comprise a DNA encoding a reporter protein wherein said DNA is operatively linked to a GPR91 responsive transcription element.
9. The method according to claim 8, wherein step (b) is carried out in the presence of increasing concentrations of at least one compound whose ability to inhibit signal transduction activity of said receptor protein(s) is sought to be determined.
10. The method according to claim 9, wherein step (c) comprises monitoring in said cells the level of expression of the reporter protein as a function of the concentration of the compound, thereby indicating the ability of said compound to inhibit signal transduction activity.
11. The method according to claim 8, wherein said GPR91 responsive transcription element is a cAMP responsive transcription element.
12. A method of screening for agonists or antagonists of GPR91 activity comprising: (a) contacting cells which express a GPR91 receptor with a candidate compound, (b) assaying a cellular response, and (c) comparing

the cellular response to a standard cellular response made in absence of the candidate compound; whereby, an increased cellular response over the standard indicates that the compound is an agonist and a decreased cellular response over the standard indicates that the compound is an antagonist.

13. The compound of the method of claim 1.

14. A method for treating, diagnosing or preventing allergic asthma in a patient comprising administering an allergic asthma treating amount of a GPR91 receptor modulator to the patient.

15. The method of claim 14, wherein the GPR91 receptor modulator is a GPR91 agonist.

16. The method of claim 14, wherein the GPR91 receptor modulator is a GPR91 antagonist.

ABSTRACT

The present invention relates to agonists and antagonists of GPR91 in Mast cells, their use in the treatment of asthma, and methods of screening therefore.